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Method of Preparation and Test for Bacterial Pyrogen*

By Henry Welch, Herbert O. Calvery, W. T. McCloskey and C. W. Price

The need for a standard method of testing intravenous solutions for demonstration of freedom from pyrogenic substances provoked a collaborative study of this problem in which the laboratories of fourteen manufacturers and those of the Food and Drug Administration and National Institute of Health have participated. Through these studies a method of test has been developed. In the present report the method of preparation of the pyrogen used, the method of test and certain immunological studies are reported. The results obtained by collaborators, all utilizing the same method of test and the interpretation of the statistical data are reported elsewhere (1).

In 1911 Wechselmann (2) demonstrated that febrile reactions resulting from intravenous injections of arsphenamine solutions could be eliminated if the solutions were properly prepared with distilled germ-free water. In the same year Hort and Penfold (3), in discussing the dangers of hot saline injections, pointed out that distillation of the water used in a sterile Jena retort followed by immediate injection was the only reliable method of insuring absence of fever following injection. The basic and extensive studies of Seibert (4, 5), Bourn and Seibert (6) and Seibert and Mendel (7) were instrumental in clarifying the pyrogen problem. Seibert and her co-workers isolated and identified an organism from distilled water and demonstrated that it was capable of producing the pyrogenic substances and that such substances were filtrable. They showed also that water distilled from a Pyrex still and used within 24 hrs. was pyrogen-free. They later identified 2 groups of organisms from river water, both of which were capable of producing pyrogenic reactions. Rademaker (8) confirmed the work of Seibert and her co-workers and gave an elaborate set of instructions as to the proper methods of preparing pyrogen-free solutions. Later Banks (9) in a careful study of the factors involved in hyperpyrexia reactions demonstrated that pyrogenic reactions were the result of a definite, specific, etiologic factor and were produced through the introduction of dead or living products of bacterial growth or cultures of *Psuedomonas scissa* or *ureae* into the blood stream. Co Tui (10) and his co-workers in 1937 described a method for removal of pyrogen from solutions containing it by filtration with an adsorption filter (Seitz

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serum pad No. 3) and concluded that the pyrogenic agent is of a particulate nature and estimated its size to be between 50 mμ and 1 μ. More recently Walter (11) in describing the basis for successful parenteral therapy points out that "There are but two requisites for a safe supply of parenteral solutions—a source of pure raw materials and centralized responsibility for cleanliness in the preparation of solutions and apparatus."

EXPERIMENTAL

METHOD OF PREPARATION OF PYROGEN

Two strains of *Pseudonomas aeruginosa* are used in the preparation of the pyrogen solution. These cultures are grown in F. D. A. (12) broth for 24 hrs. The broth cultures are then pooled aseptically in a flask and 4 cc. added to each of 30 Roux bottles (1 L. containing F. D. A. (12) agar). The area of the agar surface per bottle is approximately 28 square inches. After incubation at 37° C. for 48 hrs., the agar surface is washed with 30 cc. of sterile distilled water. The growth in this period of incubation is extremely heavy and a pellicle usually forms on the surface of the agar. The growth from the Roux bottles is pooled aseptically in flasks and filtered through four layers of gauze and one of absorbent cotton to remove agar particles. The total yield from each bottle with the water of syneresis amounts to an average of 60 cc. of suspension per bottle. After filtration the material is placed in large French square bottles containing glass beads and shaken for 5 hrs. in a mechanical shaker. As soon as the bottles are removed from the shaker, they are placed in a 37° C. incubator for a period of 6 days. Following incubation, each batch is centrifuged at high speed in an angle centrifuge, usually for a period of 30 to 50 min. at 4000 r. p. m. and the supernatant fluid filtered through Berkefeld "N" candles. After addition of sodium chloride (8.5 Gm./L.) this filtrate constitutes the stock pyrogenic solution.

Since there is no method of standardizing the concentration of the thermogenic substance in these filtrates, it seemed desirable to determine whether the total nitrogen content of each batch could be used as an index of the potency of the fever-producing material present. In a group of 9 batches prepared in an identical manner the total nitrogen ranged from 125 mg. to 145 mg./100 cc. while the average amount of total nitrogen per 100 cc. was 138 mg. In a further group of 6 batches of pyrogen made up in the same way, total nitrogen figures of 128, 144, 140, 136, 127 and 137 mg. per 100 cc. were obtained. Animal tests indicated that pyrogenic solutions of approximately equal potencies could be prepared consistently by this technique; and, although there is no evidence to show that the potency of the pyrogenic substance is associated with the total nitrogen content, it seemed advisable to utilize it as an index of the strength of the pyrogenic solution. Accordingly a series of rabbits was injected with varying concentrations of pyrogen based on total nitrogen content. It was found that when a solution containing 5 mg. total nitrogen per 100 cc. was injected into rabbits weighing from 1500 to 3000 Gm. this solution invariably produced a typical pyrogenic response. In the collaborative test referred to above, the pyrogen solution used was prepared in the manner outlined above and the solution diluted to contain 5 mg. of total nitrogen per 100 cc. of fluid.

In an attempt to determine broadly the greatest dilution of pyrogenic stock solution which would still cause a reaction in rabbits, a series of animals was injected with 3 cc./Kg. of body weight of increasing dilutions of this stock solution. It was found that the pyrogen stock solution could be diluted to 1 to 6700 before the reactions obtained were of a character similar to those obtained with the control saline injections. At concentrations greater than this the temperature rises were indicative of the presence of pyrogen and at 1:5000 the response was such as to be considered positive by the criteria established in the test.

PYROGEN TEST

The following pyrogen method is not identical to the present U. S. P. test in that the criteria for determining a positive response are based on our interpretation of the results of the collaborative assay; whereas those used in the U. S. P. test were not only based on these data, but were modified at the suggestion of several of the collaborators. In other respects the test is identical to that adopted by the U. S. P. Revision Committee.

Test Animal.—The animal used in the pyrogen test should weigh 1000 Gm. or more, and such rabbits must have been maintained for at least one week on a uniform diet and must not have lost weight during this period. The thermometer to be used should be tested to determine the time required to read maximum temperature. Animals that have not been previously used for such tests should be checked by taking four rectal temperature readings on each of the animals at 2-hr. intervals one to three days before use. The thermometer should be inserted beyond the internal sphincter and allowed to remain a sufficient time to reach maximum temperature but in no case less than 90 seconds before the reading is recorded. Obviously sick animals should be discarded and the temperature variation on the control day should not be greater than 0.8° C. Animals may be used for the test and in subsequent tests after a rest period of not less than two days, provided the control temperature reading taken on the day of the test does not vary more than 0.6° C. from the mean temperature observed on the control day. The control reading taken on the test day constitutes the normal temperature of the test animal from which a subsequent rise due to the injection of the test material may be calculated. Test animals should be kept in individual cages protected from disturbances likely to cause excitement. Particular care should be exercised to avoid exciting the animals on the day of taking the control temperatures and on the test day. Food is withheld from any animal used, beginning one hour before the first temperature reading, and no food permitted until the day's record is complete. Free access to water is allowed. The animal should be kept at uniform temperature (plus or minus 5° C.).

Conduct of Test.—The product to be tested should be warmed to approximately 37° C. and injected in 10-cc. amounts per Kg. of body weight of the rabbit intravenously through the marginal ear vein within

15 min. subsequent to the control temperature reading on the day of the test. The temperature is recorded one hour subsequent to the injection and each hour thereafter until three recordings have been made. The syringes and needles used for these injections must have been treated to render them pyrogen-free and then sterilized. Not less than five rabbits are used for the test and the test is considered positive if three or more of the five animals show an individual rise in temperature of 0.8° C. or more above the normal established for each of these animals. If only one or two of the five animals show a positive response, the test should be repeated on a second group of five additional animals. The test shall be considered positive if two of the second group of five animals show an individual rise in temperature of 0.8° C. or more above the normal established for these animals.

IMMUNOLOGICAL STUDIES

The rabbits used for pyrogen assay as pointed out in the method above, must be kept under carefully controlled conditions and be used for this work exclusively. Where numbers of solutions are tested, this necessitates a large colony of animals. Since there has been some question as to the advisability of using rabbits more than once for assaying pyrogen material, on the basis that the animals may develop a resistance to this substance on repeated injections, a study was made of the antigenic properties of a known pyrogen tested under controlled conditions.

During the collaborative assay which required a nine-week study, 1782 injections of a known pyrogen were made by the collaborating laboratories. Over the nine-week period each rabbit received 11 injections of the pyrogen and in the great majority of cases these animals showed a typical rise in temperature following injection. Lack of antigenicity of the pyrogen would appear to be borne out by these results, which are in conformity with those reported by Banks (9) who showed that rabbits did not become immune or hypersensitive to pyrogen on repeated injection. It should be noted that in 30 of more than 1700 observations following injection of pyrogen, the rise in temperature was less than 0.5° C. and in one or two of these no rise in temperature or an actual fall in temperature was observed. This lowered reactivity to the thermogenic stimulus, however, did not occur with any regularity. It seldom occurred more than once in an animal and when it did occur the animal did show a typical rise in temperature when inoculated with pyrogen three or four days later. It was of interest as well that analysis of the data obtained during the collaborative assay showed that between 80% and 90% of these observations of lowered response to injection of pyrogen occurred in the animals of 2 of the 16 collaborating laboratories.

The 20 animals injected in our laboratories with pyrogen solution eleven times over a nine-week interval were bled from the heart two weeks after the last injection and their sera tested for precipitin using the stock pyrogen solution as antigen. Similarly the sera of the 10 control animals which received during the same interval 11 injections of pyrogen-free salt solution and one of pyrogen were tested for precipitin content. As a further control on these tests, 10 normal animals were bled and their sera tested for precipitin in a similar manner. All rabbits used in this experiment were bled twice during the next four months and their sera tested for precipitin content. The results of these tests are shown in Table I, where it will be noted that the sera of all rabbits injected 11 times with pyrogen and once with pyrogen-free saline solution showed the presence of precipitin during the following four months. As a matter of fact, the 10 rabbits which received only a single injection of pyrogen all showed some precipitin when tested with the pyrogen solution used for injection. On the other hand, the sera of 10 normal untreated rabbits failed to show precipitin for the pyrogen solution used. By the end of the fourth month following injection of pyrogen, precipitin had disappeared from the sera of both groups of rabbits injected with pyrogen solution with one exception. Since pyrogenic reactions were obtained consistently in all rabbits tested with little reduction in thermogenic effect, regardless of the number of times the animals were injected, it appeared unlikely that the precipitin demonstrated could be specific for the pyrogenic substance.

TABLE I.—NUMBER RABBITS SHOWING PRECIPITINS AT VARIOUS INTERVALS FOLLOWING FINAL INJECTION OF PYROGEN

Precipitin Titer	After 2 Weeks	After 2 Months	After 4 Months
A—Rabbits receiving 11 pyrogen and 1 saline injections	(19 Rabbits)	(13 Rabbits)	(11 Rabbits)
0	2	0	10
1:10	1	0	0
1:20	4	0	0
1:40	7	0	0
1:80	3	8	0
1:160	1	4	0
1:320	1	1	0
1:640	0	0	1
B—Rabbits receiving 11 saline and 1 pyrogen injections	(10 Rabbits)	(6 Rabbits)	(6 Rabbits)
0	0	0	6
1:10	1	0	0
1:20	2	1	0
1:40	2	1	0
1:80	4	4	0
1:160	1	0	0

NOTE: As controls the sera of 10 normal untreated rabbits were tested for presence of pyrogen precipitins. None were demonstrated.

To determine whether media extractives were the source of precipitinogen present in this pyrogen solution, several Roux bottles containing F. D. A. agar were incubated for 48 hrs. *without* inoculation and to each of them 30 cc. of distilled water was added. This aqueous solution of media extractives was then shaken with glass beads in a mechanical shaker and incubated as was previously done in the preparation of pyrogen. Tests were then made with the sera from all rabbits containing precipitin. In

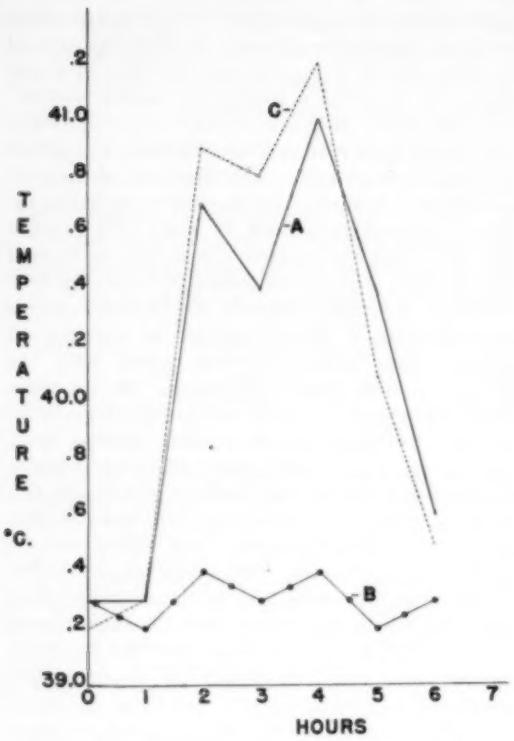

Fig. 1.—Temperature curves of rabbits injected with pyrogen solutions, media extractives and antigen-free pyrogen solutions.

A.——— Pyrogen solution—average—1728 injections.
B. °—°—° Media extractives.
C. – – – – Antigen-free pyrogen solution.

no case were we able to demonstrate a precipitinogen in this material, thus indicating that the antibody found in rabbits following injection of pyrogen solution was not due to antigenic material extracted from the media during the process of preparation.

To determine whether the sera of pyrogen-injected rabbits would remove the precipitinogen of the pyrogen solutions without adsorbing the thermogenic substance itself, rabbit sera containing the precipitin were mixed with pyrogen solution and incubated at 37° C. for 3 hrs. and the precipitate removed by centrifugation. A single adsorption was sufficient to remove all precipitinogen and although the supernatant fluid now failed to show a precipitate with rabbit sera, it produced a typical pyrogenic response on injection into normal rabbits. The rabbit precipitating sera itself on injection into normal animals was found to be nonpyrogenic. It was further found that treatment of the supernatant fluid (serum and thermogenic substance) with acetone resulted in a heavy precipitate which, although largely insoluble in water, released the pyrogenic substance when washed once with water. It would appear from these results that the pyrogenic substance is not antigenic and is not associated with the precipitin obtained on injection of the stock pyrogen solution. The thermogenic response obtained on injection of normal rabbits with these various solutions is graphically represented in Fig. 1.

To demonstrate further the lack of antigenicity of the pyrogenic substance attempts were made to free it from precipitinogen by chemical means. The following procedure was used:

To 100 cc. of stock pyrogen solution (undiluted) is added 150 cc. of ethyl alcohol. After 3 hrs. at room temperature the precipitate that forms is removed by centrifugation and discarded. The pyrogenic substance is then precipitated from the alcoholic supernatant fluid with 150 cc. of acetone (C. P.). After incubation for 24 hrs. at 37° C. the precipitate is removed and dissolved in 5 cc. of distilled water. Ten cubic centimeters of methyl alcohol (abs.) is then added. A precipitate forms and after standing at room temperature for 2 hrs., this is removed by centrifugation and discarded. This latter precipitate was shown to contain practically all of the precipitinogen originally present. After removal of the alcohol from the supernatant fluid by evaporation under reduced pressure, the pyrogenic solution is brought up to the original volume (100 cc.) with distilled water and made isotonic with sodium chloride. Titration of the thermogenic substance in rabbits showed the chemically separated material to be of approximately the same potency as the original stock pyrogenic solution. After separation of the precipitinogen and thermogenic substances by the above procedure, the sera of all rabbits showing antibody following injection of the original pyrogenic solution were tested against the chemically separated components. The thermogenic component failed to precipitate in all rabbit sera tested while the precipitinogen component showed a typical precipitin reaction in all cases.

As a final check on the lack of antigenicity of the thermogenic substance a series of five rabbits was injected twice weekly for a six-week interval with the chemically separated pyrogenic component. All rabbits exhibited a strong thermogenic response following each injection. Each rabbit was bled from the heart at the end of the third and sixth weeks and all sera tested for the presence of precipitin. None could be demonstrated.

COMMENT

The need for a standard and accurate method for the testing of intravenous solutions for thermogenic properties is undisputed; and, although the method presented in this report may need minor modifications as it receives more widespread critical usage, it appears for the present at least to give satisfactory and reproducible results.

In these studies repeated injections of a known pyrogen into rabbits failed to stimulate in these animals an immunological response to the thermogenic effect. The antibody which developed on injection of the pyrogen solution was shown not to be produced by the thermogenic substance, nor could it be demonstrated to be the result of antigenic substances extracted from the media during the process of preparation. It appears likely that the precipitin demonstrated is the result of an antigenic stimulus produced by soluble bacterial proteins not associated with the substance responsible for hyperpyrexia, since separation of the precipitinogen and thermogenic substance either by adsorption with a precipitating serum or by chemical means was easily accomplished. Lack of antigenicity was further demonstrated by our inability to produce precipitins on injection into rabbits of the thermogenic substance freed from precipitinogen by chemical means.

SUMMARY AND CONCLUSIONS

1. A method for the preparation and testing of pyrogen is presented.

2. The thermogenic substances derived from strains of *Pseudomonas* are nonantigenic.

3. Solutions of pyrogen derived from *Pseudomonas* strains contain a precipitinogen which is not associated with the thermogenic effect.

4. The precipitinogen and thermogenic substances may be separated by chemical means or by adsorption with precipitating sera produced with the stock pyrogen solution.

5. Rabbits may be used repeatedly for testing intravenous solutions for thermogenic substances.

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Results of First U. S. P. Collaborative Study of Pyrogens*

By William T. McClosky, Clifford W. Price, Walton Van Winkle, Jr., Henry Welch and Herbert O. Calvery

Because of the recognition of the production of untoward reactions by thermogenic substances sometimes present in solutions intended for intravenous use, it became apparent that a suitable test for the presence of these substances must be devised. It was highly desirable that this test should be well standardized and receive official recognition. To this end the present collaborative study was inaugurated by Subcommittee No. 3 (Biological Assays) of the Committee of Revision of the U. S. Pharmacopœia in June, 1941.

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EXPERIMENTAL

For the purposes of the study, the test was based upon the work of Seibert (1, 2), Bourn and Seibert (3) and Seibert and Mendel (4). The details of the directions submitted to the collaborators and the method to be used were as follows:

Directions.—Ten rabbits are to be used on the pyrogenic material and 5 on the physiological salt solution. The control temperatures of these animals should be taken according to the proposed method, and, for the purposes of this assay, preferably on Thursday and Friday of the immediately preceding week. The experiment should then be begun on Monday or Tuesday of the following week. Two control readings should be taken of all of the animals on the day of the test, and approximately 15 min. subsequent to the second control reading, 10 of these animals should be injected with the pyrogenic material and 5 with saline. Temperature readings should be taken according to the assay procedure and recorded on the chart. On Thursday or Friday of the same week, this procedure should be repeated exactly as above and the experiment continued for 5 weeks, using the same animals twice each week. (Since we have found that not more than 10 animals can be handled successfully by one person on the same day, it will be necessary, if one person is to handle all the animals, to divide them into 2 groups using one group on Monday and the second group on Tuesday and repeat with the Monday group on Thursday and the Tuesday group on Friday.) A rest period of 3 weeks is then permitted and the same animals are injected in the first part of the ninth week with the solutions they had previously received. In the latter part of the ninth week, the test is reversed and thus the animals which had previously received the pyrogenic material should receive saline and those which had previously received saline should receive the pyrogenic material and all temperatures should be recorded on charts.

ASSAY

Test Animals.—Use healthy rabbits weighing 1500 to 3000 Gm. which have been maintained for at least one week on a uniform diet and have not lost weight.

Before use in the test, take 4 rectal temperature readings on each of the animals at 2-hr. intervals on each of 2 successive days in order to accustom the animals to the experimental procedure and, further, to make it possible to discard those with more than 1.2° C. (2° F.) variations in temperature. Insert the thermometer beyond the internal sphincter and allow it to remain at least 2 min. before the reading is recorded. On the day of the test take 2 temperature readings 1 hr. apart before the injection of the test material. Animals may be used for the test and in subsequent tests after a rest period of not less than 2 days provided the average of the two temperature readings taken 1 hr. apart on the day of the test fall below or do not exceed by more than 0.5° C. (0.9° F.) the highest reading recorded in the original 8 readings. The average of the 2 readings taken on the test day constitutes the normal temperature of the test animal from which a subsequent rise due to the injection of the test material is calculated.

Keep test animals in individual cages protected from disturbances likely to cause excitement. Exercise particular care to avoid exciting the animals on the days of taking the control temperatures and on the test day. Withhold food from any animal used, beginning 1 hr. before the first temperature reading and permit no food until the day's record is completed. Free access to water is allowed. Keep the animals at uniform temperatures (±5° C.) during the control and test periods. They should preferably be housed in quarters maintained at constant temperature and humidity.

Dosage.—Three cubic centimeters per Kg. of body weight of the product to be tested, warmed to approximately 37° C., is injected intravenously through the marginal ear vein of the rabbit approximately 15 min. subsequent to the second temperature reading on the day of the test and hourly readings are recorded for at least 5 hrs. following the test injection. Not less than 5 rabbits shall be used for each test and the test shall be considered positive if 3 or more of the 5 animals show an individual rise in temperature of 0.8° C. (1.5° F.) or more above the normal established for each of these animals. If only 1 or 2 of the 5 animals show a positive response, the test must be repeated on a second group of 5 additional animals. The test shall be considered positive if 2 of the second group of 5 animals show an individual rise in temperature of 0.8° C. (1.5° F.) or more above the normal established for these animals.

The test material was prepared by the Division of Bacteriology of the Food and Drug Administration. The method of preparation and nature of this material have been discussed elsewhere (5). Subdivisions of this material were used by each of the sixteen collaborators.[1]

On completion of the tests, each collaborator submitted the record of his results for appraisal. Sixteen collaborators submitted reports; fifteen of which form the basis of this study. The results from one collaborator were not used since his data pertained to another phase of the general problem.

RESULTS

The fifteen laboratories made a total of 3300 tests on 253 rabbits as follows: 501 control tests during which the animals were accustomed to the procedure, 1782 tests with the pyrogenic material and 1017 tests with nonpyrogenic physiological salt solution.

[1] The following laboratories participated in this study and their help is gratefully acknowledged: Abbott Laboratories, North Chicago, Ill.; Baxter Laboratories, College Point, N. Y.; Geo. A. Breon and Co., Kansas City, Mo.; Cutter Laboratories, Berkeley, Calif.; Don Baxter, Inc., Glendale, Calif.; Food and Drug Administration, Washington, D. C.; Hospital Liquids, Inc., Chicago, Ill.; Lederle Laboratories, Inc., Pearl River, N. Y.; The Lilly Research Labs., Indianapolis, Ind.; Mulford Biological Labs., Glenolden, Pa.; National Institute of Health, Bethesda, Md.; Parke, Davis and Co., Detroit, Mich.; Schering and Glatz, Inc., New York City; E. R. Squibb and Sons, New Brunswick, N. J.; The Upjohn Co., Kalamazoo, Mich.; and Warner Institute for Therapeutic Research, New York City.

Variations in Normal Rectal Temperature of Rabbits.—The normal rectal temperature of each rabbit was observed every 2 hrs. for a period of 6 hrs. on 2 successive days before use in the test. The average maximum variation in temperature observed was 0.34 ± 0.018° C. on the first day and 0.31 ± 0.014° C. on the second day. The maximum variation in rectal temperature on either control day was not more than 0.8° C. in 97% of 253 observations nor more than 0.6° C. in 87% of the observations.

The distribution of maximum temperature readings did not differ significantly from a normal distribution with the greatest number, 14.7% of 501 observations of the readings at 39.3° C. Eighty-three per cent of the readings were between 38.7° and 39.9° C. Of the 253 rabbits used, 38, or 15%, had a maximum temperature reading on one or more of the control days in excess of 39.8° C.

On the day of the test, 2 control temperature readings were made on each animal before the injection of the test material. In all, 5648 observations of control temperature were made in the 253 rabbits on the day of the test. The maximum variation between any 2 consecutive control readings in any animal was not more than 0.5° C. in 98% of the observations and not more than 0.2° C. in 85% of the observations. Because of this small variation, it was concluded that only one observation of the control temperature need be made, from which to estimate the rise in temperature due to pyrogenic material.

The maximum control temperatures on the day of the test likewise showed a distribution which did not differ significantly from the normal. The greatest number of readings, 12.96% of 2824 observations, was observed at 39.2° C. Eighty-five per cent of the readings were between 38.8° and 39.7° C. Of the 253 rabbits used, 66, or 26%, had a maximum control temperature of more than 39.8° C. Because, in the opinion of some of the investigators, a rectal temperature of 39.8° C. or above should be considered abnormal, the restriction was inserted in the pyrogen test appearing in the U. S. P. XII that animals showing a temperature of 39.8° C. or above should not be used. Since animals having a control temperature of 39.8° C. and used in this study showed no significant difference from the other animals in their response to pyrogens, we believe that the arbitrary exclusion of such animals is unnecessary.

Response to Injection of Pyrogenic Material.—After taking the two control temperature readings on the day of test, 3 cc./Kg. of the test material was injected intravenously into rabbits. Hourly temperature readings were made for 5 hrs. thereafter. The average maximum rises in temperatures observed following the injection of the pyrogenic material are shown in Table I.

The results suggest that the animals may have become resistant to the thermogenic action of the material as the test progressed. This point requires further elucidation. Elsewhere (5) it has been shown that the substance responsible for the febrile reaction is not antigenic and this is borne out by the fact that in these tests no sensitization or anaphylactic response was noted.

In the 1587 observations made with the pyrogenic material it was found that 86.8% of the maximum temperature rises occurred within 3 hrs. following injection of the test material. Therefore, we believe there is no need to observe the animals for a period longer than this.

During the first 3 hrs., the maximum temperature rise following injection of pyrogenic material was between 1.0° and 2.0° C. in 70% of the observations. Only 1.7% of the maximum temperature

TABLE I.—AVERAGE MAXIMUM TEMPERATURE RISE (° C.) FOLLOWING PYROGEN INJECTION

Laboratory No.	1st Week		2nd Week		3rd Week		4th Week		5th Week		9th Week	Saline
PY-1941-1[a]	1.83	1.58	1.28	1.21	1.27	1.27	1.14	1.01	1.17	1.26	1.73	−0.09
PY-1941-2	2.31	2.08	1.80	1.58	1.54	1.58	1.48	1.37	2.01	1.53	1.95	0.05
PY-1941-3	2.24	2.08	1.72	1.50	1.56	1.51	1.57	1.45	1.58	1.61	1.55	0.29
PY-1941-4	2.62	2.23	2.21	1.94	1.75	1.57	2.11	1.45	1.46	1.12	1.90	0.16
PY-1941-5[b]	2.16	2.15	2.17	1.46	1.65	1.27	1.94	1.57	1.64	1.12	1.72	0.18
PY-1941-7	2.21	1.78	1.85	1.46	1.60	1.21	1.70	1.51	1.76	1.49	1.92	0.08
PY-1941-9[c]	2.10	1.65	1.47	1.26	1.42	1.50	1.63	1.78	1.81	1.88	1.96	−0.18
PY-1941-10[d]	1.87	1.96	1.90	1.68	1.70	1.66	1.54	1.36	1.42	1.45	1.83	0.18
PY-1941-11	2.02	2.36	2.18	1.92	2.04	1.77	1.95	1.67	1.99	1.00	2.08	0.19
PY-1941-12	2.38	2.14	1.47	1.64	1.25	1.52	1.47	1.46	1.50	1.64	1.70	0.37
PY-1941-13[e]	1.56	1.64	1.52	1.49	1.15	1.10	1.08	1.29	1.33	1.50	1.57	−0.11
PY-1941-14[f]	2.00	2.05	2.06	1.90	1.93	1.72	2.01	2.00	2.03	1.94	1.68	0.09
PY-1941-15	1.72	1.74	1.52	1.43	0.89	1.27	1.41	1.33	1.67	1.63	1.71	0.24
PY-1941-16A[g]	2.54	2.02	2.02	1.78	2.05	1.86	2.09	1.89	1.78	2.09	2.39	0.21
PY-1941-16B[g]	2.11	1.93	1.91	1.48	2.04	1.75	1.84	1.94	1.76	1.90	1.88	0.07
PY-1941-17	1.64	2.13	1.54	1.51	1.33	1.50	1.69	1.54	1.88	1.61	1.82	0.03
Average	2.08	1.97	1.85	1.57	1.57	1.50	1.66	1.53	1.67	1.54	1.83	0.10

[a] Five animals died during test.
[b] Control temperatures much lower (below 39.0° C.) than other laboratories.
[c] Ten animals had control temperatures above 39.8° C.
[d] Thirteen out of sixteen animals had control temperatures over 39.8° C.
[e] Five animals used in these tests developed control temperatures too high for use in subsequent tests.
[f] Ten observations on 4 rabbits revealed control temperatures high enough to cause their discard.
[g] Duplicate tests were conducted by this laboratory.

TABLE II.—AVERAGE MAXIMUM TEMPERATURE CHANGE[a] (° C.) FOLLOWING SALINE INJECTION

Laboratory No.	1st Week		2nd Week		3rd Week		4th Week		5th Week		9th Week	Pyrogen
PY-1941-1	0.08	0.35	0.25	0.18	0.21	0.30	0.02	0.09	0.04	0.13	0.24	1.29
PY-1941-2	0.15	0.24	0.12	−0.06	0.22	0.19	0.05	0.13	0.09	0.17	−0.09	1.99
PY-1941-3[b]	0.63	0.34	0.40	0.11	−0.03	0.09	0.19	−0.10	0.25	−0.27	0.46	1.63
PY-1941-4	0.17	0.18	0.11	0.56	0.38	0.35	0.35	0.14	0.25	0.37	0.06	2.53
PY-1941-5[c]	−0.16	0.16	0.12	0.13	0.26	0.01	0.31	0.08	0.27	−0.08	0.21	1.41
PY-1941-7	−0.39	−0.07	0.02	0.12	−0.25	−0.22	−0.03	−0.33	−0.14	−0.34	0.00	1.86
PY-1941-9	0.38	0.40	0.19	−0.05	0.06	0.24	−0.09	−0.19	−0.12	0.09	0.00	2.19
PY-1941-10	0.28	−0.16	0.24	0.14	0.20	0.24	0.36	0.21	−0.01	−0.04	0.13	2.17
PY-1941-11[b]	0.34	0.31	−0.01	−0.01	−0.11	0.96	0.35	−0.05	0.73	0.50	0.55	1.91
PY-1941-12[b]	1.01	0.96	0.66	0.53	0.75	0.38	0.62	0.53	0.39	0.47	0.59	1.89
PY-1941-13[d]	0.15	0.03	−0.06	−0.02	0.10	0.09	−0.13	−0.30	0.03	−0.06	0.04	1.19
PY-1941-14[b]	0.36	0.40	0.00	0.45	0.64	0.50	0.37	0.16	0.34	0.21	−0.10	2.09
PY-1941-15	0.30	0.23	0.43	0.25	−0.09	0.17	0.13	0.41	0.29	0.32	0.54	2.01
PY-1941-16[e]	0.05	−0.06	0.13	0.05	0.09	0.09	−0.04	−0.14	−0.04	−0.08	−0.05	2.15
PY-1941-17	0.11	0.11	0.12	0.09	0.13	−0.01	0.03	0.09	0.09	−0.05	0.26	2.01
Average	0.23	0.22	0.18	0.16	0.17	0.22	0.16	0.05	0.15	0.09	0.15	1.88

[a] Unless otherwise indicated all changes are plus.
[b] On one or more occasions the average temperature rise of the 5 animals indicated a false positive reaction.
[c] Four false positive reactions in 2 animals.
[d] All 5 animals, individually, gave false positive reactions, one on 4 occasions, one on 5 occasions and 3 on 6 occasions.
[e] Average of 10 animals.

rises were 0.5° C. or less. Since it has been shown in this study that the maximum normal temperature variation in rabbits is less than 0.6° C. in 87% of the cases, it appears that a temperature rise of 0.6° C. or more following injection of suspected material is indicative of the presence of pyrogens. Consequently this figure was recommended for use in the pyrogen assay incorporated in the U. S. P. XII. Using this criterion it is reasonable to expect that less than 2% of the animals would give false negative reactions. By using sufficient animals, these false reactions may be eliminated from consideration.

Response to Injection of Nonpyrogenic Material.—Tests identical to those performed with the pyrogenic material were carried out using pyrogen-free physiological salt solution. The maximum temperature changes observed following injection of this salt solution are shown in Table II.

These results show that the intravenous injection of fluid, *per se*, is not responsible for the febrile reaction. Furthermore, in 92% of the 1017 sets of observations the maximum temperature rise following injection of nonpyrogenic saline was less than 0.6° C., thus supporting the arbitrary dividing line established between positive and negative reactions discussed above. In fact, in 78% of the sets of observations the maximum temperature rise was less than 0.4° C. It is obvious that one may expect about 8% false positive reactions, these again being eliminated by the use of sufficient animals.

DISCUSSION AND SUMMARY

This first collaborative test has apparently established certain points and suggested further work on others.

The primary purpose of using a strong pyrogen over so long a period of time was to try to obtain evidence to settle the controversy that has often arisen concerning the possible sensitization of animals to a pyrogenic material. If it is true that pyrogens are the result of autolysis of microorganisms, it seemed that a pyrogen prepared from such source could be considered representative of pyrogenic materials. The pyrogen used in this collaborative test was of such strength that the injection of 3 cc./Kg. of animal would almost certainly produce a strong reaction but would rarely prove fatal. If sensitization could occur, the procedure used in this test should have been able to demonstrate such a phenomenon. As a matter of fact no such evidence was received from any one of the 15 laboratories participating. It therefore does not seem likely that sensitization is a phenomenon which results after pyrogen administrations of this strength and probably therefore not of weaker concentrations.

Another point of interest was to determine whether or not the animals become refractory and whether it would be necessary to discard them after they had shown a positive response to pyrogens. The evidence obtained in this collaborative test on this point is illustrated in Table I where it is demonstrated that the use of this strong pyrogen as often as twice per week does show a definite trend toward a lesser response

on repeated application; but, when a period of three weeks was allowed to elapse, the response was approximately equal to the initial. This fact and the fact that throughout the whole test only 1.68% of false negatives occurred, when 0.6° C. rise in temperature was used as the criterion, demonstrate that it is not necessary to discard animals which show a positive response. The length of the period between tests for complete recovery of the animal has not been established; the evidence indicates, however, that 2 days is not sufficient with this strong pyrogen.

Evaluation of the data obtained on the 2 control days with a view to determining whether or not more than 1 control day was necessary indicates that the information obtained from the 2 control days was not significantly greater than that from 1 day for the purposes of carrying out the pyrogen test. Therefore, it seems that only 1 control day is necessary.

Evaluation of the data with regard to the number of control readings required on the day of the test indicates that the amount of information obtained from 2 readings is not sufficient to justify the requirement of 2 readings on the day of the test. The criterion used in the collaborative test for discarding animals could not be used when temperatures were recorded for only 1 day and when only 1 control reading was required on the day of the test. Therefore the limit of 39.8° C. was chosen as the upper limit for temperature of animals to be used in tests. In the light of the data obtained by the collaborative study, this may be somewhat conservative. However, interpreted in such a way that it means that those animals are discarded for that particular time and may subsequently be used when and if their temperature falls below 39.8° C., the loss of animals from the colony will certainly not be considerable. This suggests the advisability of taking control temperature on more than 5 rabbits on the day of the test.

When 0.6° C. rise in temperature is used as a criterion, there were approximately 8% false positive reactions. If such a condition arises, it is obvious that less than 5 animals should not be used in testing solutions for pyrogenic activity.

The test as carried out required approximately 7 hrs. If it could be appreciably shortened without loss of accuracy and information, time and expense could be saved. The collaborative study demonstrated that it could be shortened to approximately 4 hrs. without significant loss in pertinent information. This saving of time can be obtained by eliminating one of the control readings on the day of the test and the fourth and fifth readings following the injection.

There have been many suggestions that the volume of solution used in the test animal should be related to the volume per kilogram body weight of the material used in the clinics. Although the collaborative study did not bear strictly on this point it did demonstrate that a considerable volume of a very strong pyrogen can be injected per kilogram body without injury to the animals.

In conclusion the following may be suggested as some of the problems which require further work:

1. A determination of the lowest concentration which will give a positive reaction.

2. A determination of the sensitivity of the test animal as compared to man.

3. A determination of how long a rest period should be allowed between tests.

4. A determination of whether, with very weak pyrogens, animals should be used in repeated tests.

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The Chemical Nature of Vitamin P*

By Ralph H. Higby

The therapeutic action of the flavones was studied by Akamatsu (1) and Fukuda (5) in 1931, but received no general recognition until Armentano and Szent-Györgyi (3) in 1936 announced the vitamin-like action of two crude flavone preparations from lemon peel, "citrin" and "eriodictin," in the treatment of abnormal capillary permeability and fragility.

As reported by Bruckner (4), neither of these preparations was a pure substance, citrin being a crystalline mixture of hesperidin and eriodictyol glucoside; eriodictin, the mother liquor from this crystallization, containing relatively larger amounts of eriodictyol glucoside and less hesperidin. Later Armentano (1938) (2) observed that the injection of citrin in cats caused a reduction of blood pressure. Other flavone dyes, quercitrin, naringenin, rhamnetin and 6,8-oxyflavon, reduced blood pressure, but hesperidin and eriodictyol did not.

For this reason Armentano postulated the existence of other substances in citrin which caused the blood pressure drop, and later Robeznieks (7), as a result of chromatographic adsorption studies, announced the presence of an unidentified quercitrin-like third component.

From these reports it appeared that lemon peel contains two closely associated therapeutic flavone substances, one of which, "vitamin P," controls the condition of the capillaries, the other influences blood pressure.

The investigation of vitamin P in this laboratory was commenced in 1938. A number of relatively crude preparations of the flavanone constituents of citrus peel were used and subjected to pharmacological and clinical tests. These preparations included crude orange hesperidin, lemon citrin, so-called "calcium eriodictate" from both oranges and lemons, and lemon "eriodictin." A gradual accumulation of reports indicated that each of these preparations contained in varying proportions both the blood pressure reducing factor and the capillary permeability factor.

Furthermore, it became apparent that orange derivatives, such as crude hesperidin which contains no eriodictyol, were at least as effective in controlling capillary permeability and fragility as the lemon preparations which contain considerable quantities of this substance; thus, seemingly upsetting Szent-Györgyi's belief that an eriodictyol glucoside was the active substance.

EXPERIMENTAL

The problem was then to isolate the active components of these crude preparations in pure form. This was greatly complicated by the tendency of the flavanones to crystallize in complexes such as "citrin," which give relatively sharp melting points and otherwise resemble pure substances. In the course of this investigation eriodictyol (m. p. 267° C.) and limonin (m. p. 292° C.) were isolated for the first time from lemon peel. Relatively pure eriodictyol glucoside was also isolated in crystalline form. This substance is extremely water-soluble but crystallizes readily from anhydrous isopropanol or *n*-butanol as sphero-crystals. These revert to an amorphous glass on heating, and no definite melting point could be obtained. The glucosidal nature of this compound was indicated by the formation of an osazone, and acid hydrolysis yielded eriodictyol.

The resolution of 3 active crude preparations into their components is shown in Table I. The pharmacological testing of these fractions was done by Dr. C. H. Thienes and his associates in the School of Medicine of the University of Southern California. Test methods and detailed results will be reported by them independently.

They found that pure hesperidin administered as a suspension of needles in saline solution, pure limonin, and pure eriodictyol from eriodictyol glucoside had no effect on either blood pressure or upon capillary fragility. Eriodictyol glucoside reduced blood pressure only slightly and was ineffective in preventing lung hemorrhages in mice.

Their tests on the water-soluble yellow pigment from crude orange hesperidin showed it to be active in reducing blood pressure.

This material on purification crystallized from absolute isopropanol as sphero-crystals. It was soluble in water, alcohol and acetone, and somewhat soluble in ether. Acetone solutions gave strong

* Received November 25, 1942, from the Research Department, California Fruit Growers Exchange, Ontario, Calif.

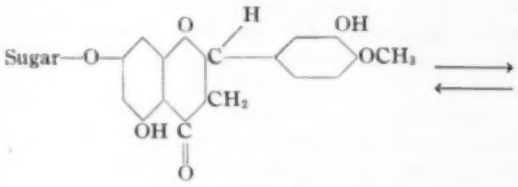
TABLE I.—COMPONENTS OF CRUDE VITAMIN P PREPARATIONS

Material Designated as	Source	Components
Crude hesperidin	Orange peel	Hesperidin (m. p. 261–262° C.). Water-soluble yellow pigment. Inert pectinous material
Citrin	Lemon peel	Hesperidin (m. p. 261–262° C.). Limonin (m. p. 291–292° C.). Eriodictyol glucoside. Water-soluble yellow pigment
Eriodictin	Lemon peel	Hesperidin (m. p. 261–262° C.). Limonin (m. p. 291–292° C.). Eriodictyol glucoside. Water-soluble yellow pigment

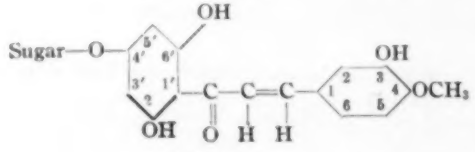
colors with the Wilson boric acid test, indicating flavone or chalcone structure. Water solutions on standing slowly deposited hesperidin crystals.

These properties led to the belief that the yellow pigment was hesperidin chalcone. This substance is described in the literature only by Wilson (10), who obtained a yellow water-soluble substance which did not crystallize. The chalcones as a class, however, have been extensively investigated, and many of their properties are known. Typically, they are unstable, being formed from flavanones by the action of mild alkali and reverting to this form in acid solution or when heated.

The mechanism of this formation and reversion is shown in the following equation



Hesperidin



Chalcone

To establish the identity of the yellow pigment from crude hesperidin with hesperidin chalcone (2′,3,6′-trihydroxy, 4-methoxy chalcone glucoside), this substance was prepared from pure hesperidin (m. p. 261–262° C.) by a modification of Wilson's method which allowed crystallization of the chalcone from anhydrous butanol.

Method.—Five grams of pure hesperidin is weighed into a 250-ml. flask and covered with 20 ml. of petroleum ether. Fifty milliliters of 1.0 *N* sodium hydroxide is then introduced carefully to form a layer beneath the petroleum ether, which latter serves to prevent atmospheric oxidation of the chalcone. The flask is then stoppered, using a rubber stopper equipped with a Bunsen valve, which allows vapors to escape but no entrance of air. The flask is then placed in a cold water bath and heated on a hot plate. Timing is started when the water bath begins to boil, and heating continued for 30 min. The flask is cooled, 50 ml. of *n*-butanol added, then immediately 50 ml. of 1.0 *N* HCl to exactly neutralize the solution. The flask is then shaken thoroughly, the contents transferred to a separatory funnel, and the butanol layer which contains most of the chalcone is separated. The extraction is repeated with a second 50-ml. portion of butanol. The butanol extracts are combined and washed twice with 25-ml. portions of distilled water to remove salt. The butanol extract is then filtered and concentrated under vacuum until crystallization commences. This is never complete, but the chalcone remaining in solution can be precipitated by the addition of 2 volumes of petroleum ether. The precipitate is filtered off, washed with ethyl ether, and dried in a vacuum desiccator.

The substance prepared in this way is similar in all respects to the yellow pigment from crude hesperidin. It is a deep yellow powder, soluble in water, alcohol or acetone. In acid solution it rapidly reverts to insoluble hesperidin. It gives a brilliant yellow color in the Wilson boric acid reaction and little or no cyanidin color in the flavanone test if the reaction mixture is cooled to prevent hesperidin formation. Recrystallization from absolute isopropanol yields sphero-crystals which sinter to an amorphous yellow resin and decompose at 110–112° C. Pharmacological tests of the chalcone showed it to be active in reducing blood pressure and in decreasing capillary fragility.

The hesperidin from which this chalcone was prepared had been previously tested pharmacologically and found inactive. Thus insoluble, inactive pure hesperidin was activated merely by solubilization. Apparently pure hesperidin, because of its extreme insolubility, is not assimilable, and hence inactive; but hesperidin *in soluble form* can function as vitamin P.

The soluble forms of hesperidin are rather limited. It occurs in solution in the cells of the fruit, possibly as the chalcone, possibly as the glucose-hesperidin complex hypothesized by Hall (6). The alkali and alkaline-earth salts of hesperidin are readily soluble but give *p*H values of 11.0 or higher. The chalcone alone remains as a relatively neutral water-soluble form of this substance. It was found, however, that hesperidin chalcone reverted easily to hesperidin on heating in neutral water solution or even on long storage in the dry state, and hence was too unstable to be a desirable pharmaceutical.

To avoid this reversion and loss of solubility, it was suggested by C. W. Wilson that ring closure might be permanently prevented by methylation of the hydroxyl group in position 6′ formed when the pyrone ring is split. This was apparently accomplished by simultaneously chalconizing and methylating, using a limited amount of methyl sulfate, and taking advantage of the nascency of the newly formed hydroxyl to obtain selective methylation.

The resulting product was a deep yellow powder, readily soluble in water, quite soluble in alcohol and acetone and slightly soluble in ether. No reversion to hesperidin occurred in neutral solution, or in solutions acidified to *p*H 3.0. This substance gave a striking depression of blood pressure in the pharmacological tests and was effective in reducing capillary fragility.

At the present time this compound appears to have many desirable properties, but it should not be considered to be the ultimate answer to the vitamin P problem. A great deal of experimental work is still required with this and similar derivatives of hesperidin to establish their effectiveness and nontoxicity.

No undesirable side effects have been observed from the administration of hesperidin, or citrin. Armentano, however, observed general malaise, fever and dypsnea after the intravenous clinical administration of quercetin and rhamnetin, substances which have the same double bond linkage as hesperidin chalcone. For this reason clinical testing of the methyl chalcone and other derivatives should be cautious until nontoxicity has been established.

In the light of our experience with hesperidin and hesperidin chalcone, certain assumptions made by Wawra and Webb (9) in a recent paper dealing with vitamin P appear misleading.

For example, the authors state that they "were led to regard the yellow eriodictin of Szent-Györgyi as hesperidin chalcone." This preparation was reported by Bruckner to contain eriodictyol glucoside, hesperidin and a third unidentified substance. A similar preparation made in this laboratory yielded hesperidin, eriodictyol glucoside, limonin and the yellow pigment previously described. Hence the claim that "eriodictin" is the chalcone of hesperidin is apparently erroneous.

A second questionable point is the preparation and properties of hesperidin chalcone as described by Wawra and Webb. So far as can be ascertained this substance has previously been described in the literature only by Wilson (10) who obtained a water-soluble orange powder. Using a modification of Wilson's method the writer obtained the similar material described earlier in this paper.

Hence the yellow water-insoluble preparation (m. p. 251–252° C.) described by Wawra and Webb can scarcely be hesperidin chalcone.

It can be readily shown that when pure hesperidin (m. p. 261–262 C.) is dissolved in an excess of sodium hydroxide and reprecipitated by neutralization according to the procedure described by these authors, the resulting crystals will vary in both color and melting point depending on time of contact with the alkali, *p*H of precipitation and exposure to oxidation.

To demonstrate this point the following experiment was performed; Five-tenth gram portions of pure hesperidin were dissolved in 10-ml. portions of 1.0 *N* sodium hydroxide solution, allowed to stand at room temperature and reprecipitated under various conditions. The precipitates were washed with acetone and ether and dried in a vacuum desiccator.

Results are shown in Table II.

The variation is apparently due to contamination of the precipitated hesperidin with more or less partially oxidized chalcone. That these crystals are not pure, but a mixture, can be demonstrated by dissolving the crystals in pyridine and treating the solution with decolorizing carbon. The colored chalcone is adsorbed, leaving the solution practically colorless. Dilution with water yields a precipitate of pure hesperidin.

It is also probable that rhamnose and glucose, which make up more than half the weight of the hesperidin molecule, are responsible for the oxidation-reduction effects described by Wawra and Webb. It is well known that these sugars in alkaline solution are readily oxidized by atmospheric oxygen.

These questions relate almost exclusively to the interpretation of chemical data. One important fact remains. The results of the pharmacological tests (8) indicate that Wawra's "chalcone," dissolved in a minimum amount of sodium hydroxide before administration, was very effective in reducing

TABLE II.—VARIATION IN HESPERIDIN PRECIPITATES DUE TO PRECIPITATION CONDITIONS—TIME EXPOSED TO ALKALI, AND OXIDATION

Sample	Treatment	Color	M. P., ° C.
1	Precipitated after 15 min. exposure to alkali in stoppered flask. Neutralized to *p*H 6.0	Yellowish white	252–253
2	As above, but allowed to stand 1 hr.	Yellow	247–248
3	As above, but allowed to stand $2^1/_2$ hrs.	Pale yellow	246–248
4	As above, but allowed to stand 4 hrs.	Yellowish white	248–250
5	Dissolved in open flask, allowed to stand 15 min., then pptd. at *p*H 8.0	Almost white	252–253
6	As above, but pptd. at *p*H 6.0	Pale yellow	253–254
7	As above, but pptd. at *p*H 2.0	Deep yellow	243
8	Protected from oxidation by inert gas, pptd. at *p*H 6.0	Almost white	257.5

capillary fragility. Thus hesperidin, dissolved in alkali, precipitated by neutralization, and redissolved in alkali, was the active compound. Disregarding the intermediate precipitation step, which does not seem important, the actual material was purely a solution of hesperidin in alkali.

This supports the author's original contention that the apparent inactivity of pure hesperidin is due to its extreme insolubility. When solubilized sufficiently to permit assimilation it can function as vitamin P.

SUMMARY

An accumulation of evidence indicates that hesperidin in soluble form can function as vitamin P.

The soluble forms of hesperidin are discussed. These include the natural soluble form as it exists in citrus fruit, the sodium, calcium and other alkali and alkaline-earth salts, which exist only at *p*H values of 11.0 or higher, and the chalcone. This isomer of hesperidin is water-soluble, and would be highly desirable for pharmaceutical use were it not for its ease of reversion to insoluble hesperidin. Limited methylation of the chalcone to form a methoxyl group at position 6′ prevents this reversion, causing the compound to be permanently water soluble in the desirable *p*H range.

Preliminary tests indicate that the methyl chalcone (probably 2′,3-hydroxy, 4,6′-methoxy chalcone glycoside) affords protection against capillary fragility and is effective in lowering blood pressure. Further pharmacological tests are necessary to determine possible toxicity, and to confirm the physiological activity.

Consideration is given to the recent paper of Wawra and Webb who are not in agreement with the present writer regarding the nature and properties of hesperidin chalcone.

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Absolute from Coffee Flowers*

By Noemi G. Arrillaga

Absolutes, highly concentrated forms of natural floral perfumes, are greatly esteemed by the perfumer because of the delicacy and roundness they impart to synthetic blends.

At the present time the scarcity of natural floral odors from Southern France is leading to a liberal use of synthetics which give originality and a certain amount of fixative power, but which lack the tenacity and natural note achieved by the use of absolutes. As reported by the author in 1941 (1), the possibility of using coffee flowers in the preparation of a flower absolute was tried with favorable results.

This season another opportunity to work with coffee flowers was presented, and approximately 100 pounds of coffee flowers like those shown in Fig. 1 were obtained and utilized in various experiments in the preparation of absolute.

Records kept on the picking of the flowers showed that they could be obtained at the rate of about 1 pound per man-hour. The average extraction of oil is about 0.5%.

On this basis, if the absolute commands a price commensurate with other flower

* Received November 24, 1942, from the Puerto Rico Experiment Station, U. S. Department of Agriculture, Mayaguez, P. R.

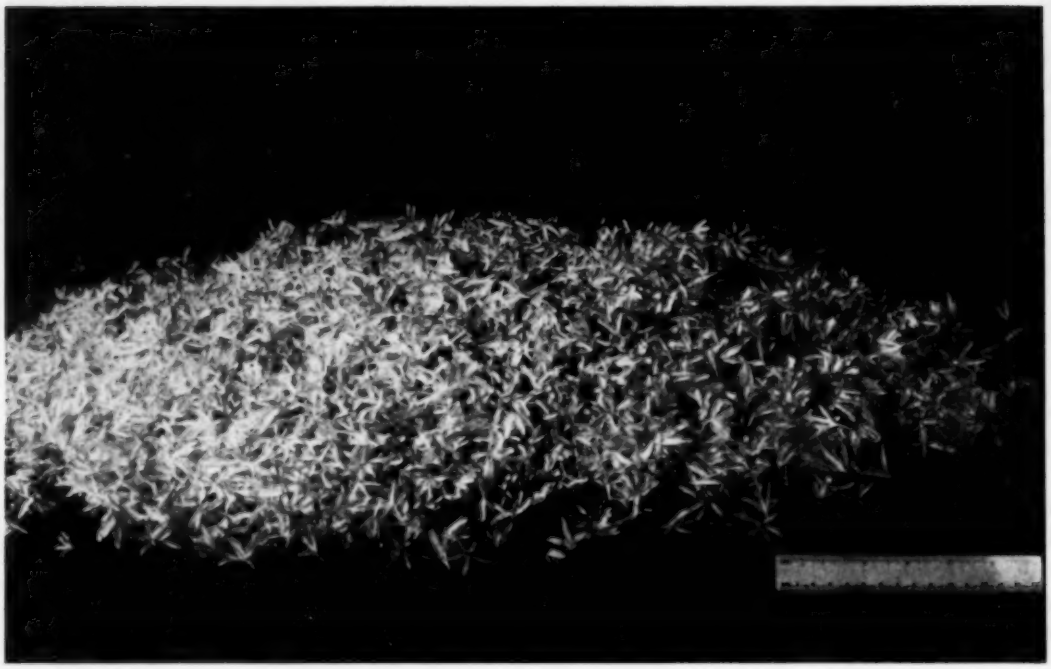

Fig. 1.—Five Pounds of Coffee Blossoms Cleaned prior to Extraction of the Absolute ($\times 1/5$).

absolutes, the product should have definite commercial possibilities.

The commercial production of this absolute would not only help the perfume trade by affording a suitable substitute for other indispensable absolutes, but also would help to relieve the excess production of coffee in the Western Hemisphere.

EXPERIMENTAL

Methods of Extraction.—It has been reported that extraction of coffee flowers with petroleum ether gave a higher yield of absolute than extraction by enfleurage (1).

Another experiment was made this past season using flowers of the Puerto Rican variety of *Coffea arabica* L. as well as imported coffee varieties. Three methods of extraction were compared. The first method consisted of maceration of the blossoms with petroleum ether in an apparatus in which the solvent was heated to 40° C. in a side chamber; the second method was maceration in liquid-petrolatum from which the absolute was recovered by successive extraction with alcohol. Soxhlet extractors involving automatic extraction with pure solvent were used in the third method.

In the volatile solvent extractions the petroleum ether was evaporated at low temperature, the concrete was dissolved in alcohol and the waxes were removed by filtering, chilling and refiltering. The alcohol was removed at low temperature to leave the absolute.

After maceration of the blossoms in liquid petrolatum, the absolute was extracted with ethyl alcohol and recovered as above.

The yields of concrete and absolute and the quality obtained by each process are shown in Table I.

It can be seen from Table I that good extraction was obtained with petroleum ether in all three apparatuses, while the rendering of the oil by maceration in liquid vaseline gave a low yield of poor quality oil.

In all cases the aroma of the absolute from the native variety was more agreeable than that from foreign varieties. Foreign varieties yielded slightly more concrete but less absolute.

Use of Different Solvents in the Extraction of Coffee Absolute.—Petroleum ether is considered the best solvent and has been most commonly used for the extraction of flower absolutes, but, during the present emergency, it is very scarce if not unobtainable.

An experiment was carried out to choose an obtainable solvent which could be used as a substitute for petroleum ether and have as many qualities as possible of the ideal solvent for the extraction of flower essences.

Ethyl ether was tried because of its resemblance to petroleum ether in solvent properties. Acetone, although miscible with water and having high solvent power for coloring matter, was chosen, because at the time it was manufactured and readily obtainable in Puerto Rico.

Coffee flowers were extracted in Soxhlet extractors with the 2 solvents and petroleum ether for comparison. The results of the experiment are shown in Table II.

TABLE I.—COMPARISON OF DIFFERENT METHODS AND TYPES OF EQUIPMENT FOR THE EXTRACTION OF COFFEE BLOSSOMS

Variety of Flowers	Type of Equipment	Yields, %		Quality	
		Concrete	Absolute	Color	Aroma
Puerto Rican	Large macerator, petroleum ether	0.836	0.665	Light brown	Good—strong
Foreign	Large macerator, petroleum ether	0.853	0.502	Light brown	Good—weak
Puerto Rican	Small macerator	0.703	0.529	Light brown	Good—strong
Foreign	Small macerator	0.859	0.500	Light brown	Good—weak
Puerto Rican	Liquid petrolatum	...	0.247	Light brown	Rancid
Foreign	Liquid petrolatum	...	0.233	Light brown	Rancid
Puerto Rican	Soxhlet, petroleum ether	0.840	0.631		Good
Foreign	Soxhlet, petroleum ether	0.873	0.563		Good

TABLE II.—YIELD AND QUALITY OF COFFEE FLOWER ABSOLUTE OBTAINED BY EXTRACTION WITH VARIOUS SOLVENTS

Solvent	Yields, %		Quality	
	Concrete	Absolute	Color	Aroma
Petroleum ether	0.8728	0.5630	Light brown fluid	Good concentrated characteristic
Ethyl ether	0.7009	0.5033	Dark brown, thick	Delicate flowery odor
Acetone	0.5394	0.1997	Very dark brown	Impure, strong odor

It can be seen from the table that the best yield of good quality absolute was obtained by extraction with petroleum ether. Although extraction with ethyl ether gave a somewhat lower yield of oil, the absolute had a superior aroma, devoid of the oily, waxy smell so commonly present in flower absolutes. Extraction with acetone was incomplete, probably due to the dilution of the solvent with the water from the flowers. The absolute obtained from this extraction was viscous and very dark. If this solvent were to be used, the absolute would have to be further purified.

Treatment of Coffee Flowers Previous to Extraction.—An experiment was carried out to find whether some method of pretreatment of coffee flowers could be devised which would result in a higher yield of absolute. One way in which a pretreatment might increase the yield would be to activate certain enzyme mechanisms leading to the production of the desired essences in the flower. This is known to be the case in jasmine and tuberose (2). Another effect of pretreatment might be purely mechanical in that the cell structure would be ruptured and the oil made more available.

Four 1-pound samples of coffee blossoms were subjected to the following treatments: 20 pounds pressure for 30 min.; desiccation with calcium chloride for 24 hrs.; freezing at −2° C. for 24 hrs.; and no treatment. All samples were extracted in Soxhlet extractors with petroleum ether for 24 hrs. The freezing treatment gave a yield slightly greater than that from the untreated flowers, while the pressure and desiccation treatments gave considerably lower yields. All of the treatments produced an absolute of good aroma. Although the increase in yield from the freezing treatment was probably not significant, it offers a possible method for storing and holding the flowers for short periods of time after picking and previous to extraction.

SUMMARY

1. Several experiments were conducted to determine the commercial possibilities of coffee flower absolute.

2. Extraction of coffee flowers with petroleum ether gave a higher yield of absolute than extraction by enfleurage and maceration with liquid petrolatum.

3. Petroleum ether gave the best yield of good quality oil among the several solvents tried. Ethyl ether gave a somewhat lower yield of oil with a superior aroma.

4. Coffee flowers frozen previous to extraction yielded slightly more oil than flowers not so treated. Pressure and desiccation treatments gave considerably lower yields. Although the increase in yield from the freezing treatment was probably not significant, it offers a possible method for storing and holding the flowers until such time as is convenient to extract them.

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(2) Poucher, W. A., "Perfumes, Cosmetics and Soaps," Fifth edition, Vol. II, D. Van Nostrand Company, New York, New York, 1936, p. 40.

Colloidal Silver Sulfonamides*

By Milton Wruble

Colloidal nonstaining silver halide preparations are still widely used, particularly in the field of otolaryngology and urology. This is true in spite of the fact that newer synthetic drugs have, in some cases, been developed for the treatment of specific ailments in these branches of medicine. To be useful, colloidal preparations of this type should possess the following properties:

1. They should not stain tissue, clothing, linen, etc., as contrasted to the dark staining silver proteins.
2. They should have colloidal properties.
3. They should be easily reversible after they are prepared and dried.
4. They should be non-irritating.
5. They should remain fairly light-resistant.
6. They should be active.

None of this class of preparations already on the market fulfills all of these requirements.

The external use of the sulfonamide group of drugs in wounds and in infections of the eye, ear, nose, sinuses, etc., has become of considerable and widespread interest. A year ago Turnbull (1) demonstrated that a 5% solution of the sodium salt of sulfathiazole brought definite relief in chronic sinusitis when used as a nasal spray and also produced dramatic results in conjunctivitis and in ear infections. However, several instances have been recorded in the medical literature of extensive tissue damage and even necrosis produced by the high alkalinity of the sodium sulfathiazole. A recent paper by Osgood (2) states in this connection:

"Sodium sulfathiazole should not be used locally, since it has no advantages over sulfathiazole powder used as described and it is so alkaline that it is likely to produce extensive tissue damage."

* From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Presented to the Scientific Section of the A. PH. A., Denver meeting, 1942.

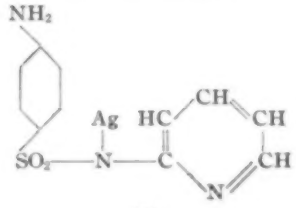
[1] The pharmacological studies were carried out by Mr. Marvin L. Pabst and the bacteriological data were furnished by Mr. Fred Mantele, both of The Upjohn Laboratories.

EXPERIMENTAL[1]

Preparation.—Colloidal silver preparations of sulfanilamide, sulfathiazole, sulfapyridine and sulfadiazine were made in this study, although the greater portion of this work is confined to the study of silver sulfathiazole. When aqueous solutions of the sodium salts of these sulfonamides and silver nitrate are added to each other in stoichiometrical proportions, the silver sulfonamides are precipitated as dense snow-white, somewhat gelatinous colloidal precipitates. No attempt was made during their preparation to guard them from excessive daylight as it was desirable to learn just what their resistance in this respect would be. With the exception of silver sulfanilamide, which darkens considerably and is thus rendered unfit for use, the others remain practically light-resistant. Their structural formulas are the following:

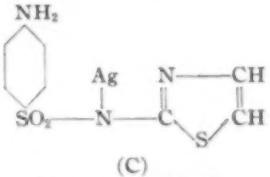
(A)

Silver Sulfanilamide



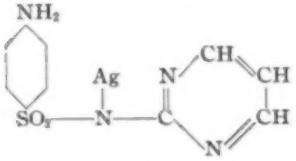
(B)

Silver Sulfapyridine



(C)

Silver Sulfathiazole



(D)

Silver Sulfadiazine

While freshly precipitated silver sulfonamides are somewhat gelatinous and colloidal in themselves, it

TABLE I.—ANTISEPTIC TEST[a, b]

Product I		Product II		Product III		Silver Sulfathiazole Compound	
Strength of Preparation, %	Width of Zone of Inhibition, Mm.	Strength of Preparation, %	Width of Zone of Inhibition, Mm.	Strength of Preparation, %	Width of Zone of Inhibition Mm.	Strength of Preparation, %	Width of Zone of Inhibition, Mm.
20	3.0	20	4	20	5.5	20	4.5
10	2.0	10	4	10	5.5	10	4.5
5	0.0	5	4	5	5.0	5	4.5
2	0.0	2	4	2	5.0	2	4.5
1	0.0	1	4	1	4.5	1	4.5

a Culture used: *Staphylococcus aureus* "209," Dept. of Agriculture, 4, p. 41 (1-90-15°).
b Method: F. D. A. Agar Cup Plate.

is necessary to further disperse the particles and keep them in such colloidal state by precipitating these salts in the presence of protective colloids, such as acacia, tragacanth, gelatin, pectin, etc. Sugars, such as beta-lactose, sucrose, dextrose, etc., when incorporated into these products, reduce their gummy characteristics when dry, and also materially aid their reversibility.

Further processing through a suitable colloid mill is advantageous for the production of a better colloidal dispersion. The precipitation of the product is then carried out with a suitable solvent such as alcohol or acetone and the precipitate is dried under reduced pressure at a temperature not to exceed 75–80° C. It is then milled to a fine granular powder.

Solutions of these compounds, up to 25%, are easily prepared in the cold by vigorous agitation and up to 40% with the aid of heat. Colloidal Silver Sulfathiazole Compound contains approximately 25% of silver sulfathiazole and a silver content of about 8%. A 20% solution has a *p*H of 5.94, which is within the range of maximum effectiveness according to recent work by Fabricant (3). In this connection the author states.

"It is therefore suggested that during acute rhinitis and acute rhinosinusitis the employment of a silver preparation which lowers the high *p*H to a level between 5.5 and 6.5—approximating the normal *p*H status of nasal secretions *in situ*—is most desirable. An ideal silver preparation, no less than a proper nasal vasoconstrictor, should not only restore and maintain normal ciliary activity but also be slightly acid with a *p*H value between 5.5 and 6.5."

It should be noted, too, that such a solution will not produce tissue damage as in the case with sodium sulfathiazole.

The bacteriological activity, toxicity, irritation and other biological tests were made on colloidal silver sulfathiazole. The others are now being investigated in the same manner but no data are as yet available.

Bacteriological Activity.—The F. D. A. Agar Cup Plate Method, using *Staphylococcus aureus*, was employed to test the antiseptic properties of this preparation. The tables indicate a comparison between this product and three competitive products already on the market. (Table I.)

Because some of these competitive products lose their antiseptic activity in the presence of blood serum, it was considered of interest to make a similar comparison using the F. D. A. Serum Agar Cup Plate Method with *Staphylococcus aureus*. The tables indicate the results obtained. (Table II.)

The U. S. P. X Antiseptic Test employing yeast was likewise used to determine its antiseptic activity as compared with the same competitive products. (Table III.)

Toxicity.—Comprehensive studies of acute and chronic toxicity of colloidal silver sulfathiazole on rats, when compared to silver proteins and to competitive nonstaining silver preparations, indicate that colloidal silver sulfathiazole is, in general, no more toxic than these.

The toxicity of silver sulfathiazole was compared with the toxicity of sulfathiazole itself by acute and chronic tests in the rat. In chronic toxicity tests in which the substances were administered orally by stomach tube to rats for a period of 14 days, the toxicity of silver sulfathiazole was greater than that of sulfathiazole. Acute oral toxicity tests are not entirely satisfactory because of the insolubility of the substance and poor absorption from the gastrointestinal tract. However, these tests seemed to indicate that silver sulfathiazole, when given orally to rats in the doses required under these tests, appears to be as toxic or more toxic than sulfathiazole.

TABLE II.—ANTISEPTIC TEST[a, b]

Product I		Product II		Product III		Silver Sulfathiazole Compound	
Strength of Preparation, %	Width of Zone of Inhibition, Mm.	Strength of Preparation, %	Width of Zone of Inhibition, Mm.	Strength of Preparation, %	Width of Zone of Inhibition, Mm.	Strength of Preparation, %	Width of Zone of Inhibition, Mm.
10	1.0	10	2.5	10	5.5	10	4.0
5	0.5	5	2.5	5	4.5	5	4.0
1	0.3	1	2.5	1	3.0	1	4.0

a Culture used: *Staphylococcus aureus* "209," Dept. of Agriculture, 9, p. 39 (1-90-10°).
b Method: F. D. A. Serum Agar Cup Plate.

TABLE III.—ANTISEPTIC ACTION OF SILVER SALTS ON YEAST CELLS[a,b]

Product I			Product II			Product III			Silver Sulfathiazole Compound		
Strength of Preparation, %	Amount of Preparation Tested, Cc.	Amount of Preparation Tested, Mg.	Amount of Gas Formed, in Cc.	Strength of Preparation, %	Amount of Preparation Tested, Cc.	Amount of Preparation Tested, Mg.	Amount of Gas Formed, in Cc.	Strength of Preparation, %	Amount of Preparation Tested, Cc.	Amount of Preparation Tested, Mg.	Amount of Gas Formed, in Cc.	Strength of Preparation, %	Amount of Preparation Tested, Cc.	Amount of Preparation Tested, Mg.	Amount of Gas Formed, in Cc.
20	2.0	400	10.0+	20	2.0	400	7.5	20	2.0	400	4.0	20	2.0	400	0.0
	1.6	320	10.0+		1.6	320	10.0+		1.6	320	3.0		1.6	320	0.0
	1.2	240	10.0+		1.2	240	10.0+		1.2	240	3.3		1.2	240	0.0
	1.0	200	10.0+		1.0	200	10.0+		1.0	200	2.6		1.0	200	0.0
	0.8	160	10.0+		0.8	160	9.5		0.8	160	2.3		0.8	160	0.0
	0.6	120	10.0+		0.6	120	8.5		0.6	120	2.5		0.6	120	0.0
	0.4	80	10.0+		0.4	80	10.0+		0.4	80	3.0		0.4	80	0.5
	0.2	40	10.0+		0.2	40	9.0		0.2	40	Bubble		0.2	40	2.5
5	2.0	100	10.0+	5	2.0	100	8.5	5	2.0	100	2.0	5	2.0	100	0.0
	1.6	80	10.0+		1.6	80	9.5		1.6	80	2.5		1.6	80	1.0
	1.2	60	10.0+		1.2	60	9.5		1.2	60	1.6		1.2	60	1.0
	1.0	50	10.0+		1.0	50	9.0		1.0	50	1.3		1.0	50	2.0
	0.8	40	10.0+		0.8	40	7.5		0.8	40	0.5		0.8	40	2.0
	0.6	30	10.0+		0.6	30	9.0		0.6	30	Bubble		0.6	30	2.5
	0.4	20	10.0+		0.4	20	8.5		0.4	20	Bubble		0.4	20	5.5
	0.2	10	10.0+		0.2	10	10.0+		0.2	10	0.5		0.2	10	8.5

[a] Culture used: Compressed Yeast (Fleischmann).
[b] Method: U. S. P. X.

Irritation.—Irritation was studied in the eye nasal mucosa, accessory sinuses, nasal cilia, bladder and urinary tract of rabbits. Colloidal silver sulfathiazole is non-irritating and compares favorably with all the other silver preparations of this type now in use by the medical profession. It does not disturb ciliary activity.

CONCLUSIONS

1. Colloidal silver preparations of sulfanilamide, sulfathiazole, sulfapyridine and sulfadiazine have been prepared. With the exception of the silver sulfanilamide, which darkens rapidly, the others remain as white or cream-white powders, easily dispersible in water.

2. Solutions of colloidal silver sulfathiazole have a slightly acid *p*H, which is in the range of maximum effectiveness according to the most recent investigations.

3. Solutions of colloidal silver sulfathiazole are non-irritating in the eye, nasal mucosa and accessory sinuses, as well as in the urinary tract of rabbits.

4. Colloidal silver sulfathiazole has been shown to be no more toxic than similar preparations already in use by the medical profession for many years.

5. Colloidal silver sulfathiazole possesses bacteriostatic properties and is mildly antiseptic. It does not lose this activity in the presence of blood serum.

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(2) Osgood, E. E., *Surg. Gynecol. Obstet.*, 75 (1942), 21.

(3) Fabricant, N. D., *Arch Otolaryng.*, 34 (1941), 302.

ANNOUNCEMENT

The following types of used laboratory equipment are urgently needed by the Board of Economic Warfare for its experimental laboratories abroad:

1. Polarimeters.
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Syntheses of Compounds Related to Lysergic Acid*

By Hurbert W. Murphy† and Glenn L. Jenkins‡

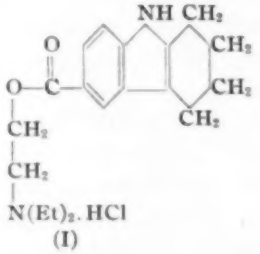
It has been confirmed that lysergic acid is the essential pharmacodynamic component of the alkaloids of ergot (1) and its chemical structure has been rather definitely established by the researches of Jacobs, Craig, *et al.* (2). Since relatively simple derivatives possessing an effective pharmacological action can be prepared from this acid obtained by special methods from the less desirable alkaloids of ergot (3), the synthesis of lysergic acid itself or of more easily prepared compounds capable of stimulating the uterine musculature presents a problem of considerable interest.

The present investigation was undertaken in an attempt to develop satisfactory methods for the preparation of certain tetrahydrocarbazole and indole derivatives for pharmacological investigation. In order to determine the applicability of the Fischer method for the synthesis of indole derivatives containing a carboxy group linked with the benzene ring of indole, the attempted synthesis of 2-methyl-5-indolylcarboxylic acid and 3-ethyl-5-indolylcarboxylic acid from acetone (*p*-carboxyphenyl)hydrazone and *n*-butanal (*p*-carboxyphenyl)hydrazone, respectively, was undertaken. Neither of the desired products could be isolated from the reaction mixture whenever several modifications of the Fischer method were employed (4). Acetone (*p*-carboxyphenyl)-hydrazone underwent ring closure and simultaneous decarboxylization in the presence of anhydrous zinc chloride to yield 2-methylindole.

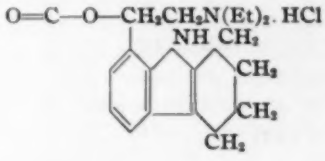
Four isomeric (5, 6, 7 and 8) 1,2,3,4-tetrahydrocarbazolylcarboxylic acids were prepared by the method of Plant (5). Attempts to convert the 5, 6 and 7 isomers to the acid chlorides by treatment with thionyl chloride alone or in the presence of pyridine (6) were unsuccessful. The hydrochlorides of the β-diethylaminoethyl esters of 1,2,3,4-tetrahydro-6- (I) and 1,2,3,4,-tetrahydro-8-carbazolylcarboxylic acids (II) have been prepared, by the method of Horenstein and Pählicke (7), for pharmacological investigation.

2-Chloro-1-cyclohexanone was prepared, but all attempts to obtain either the (*p*-carboxyphenyl)hydrazone or the (*o*-carboxyphenyl)hydrazone for further syntheses were unsatisfactory. This may have been due to the peculiar lability of the chlorine atom.

Camphor (*p* - carboxyphenyl)hydrazone and camphor (*o*-carboxyphenyl)hydrazone were prepared, but could not be converted to 1,2,3,4 - tetrahydro - 1 - methyl-1,4 - (dimethylmethylene)-6- and 1,2,3,4-tetrahydro - 1 - methyl - 1,4 - (dimethylmethylene) - 8-carbazolylcarboxylic acid.



(I)



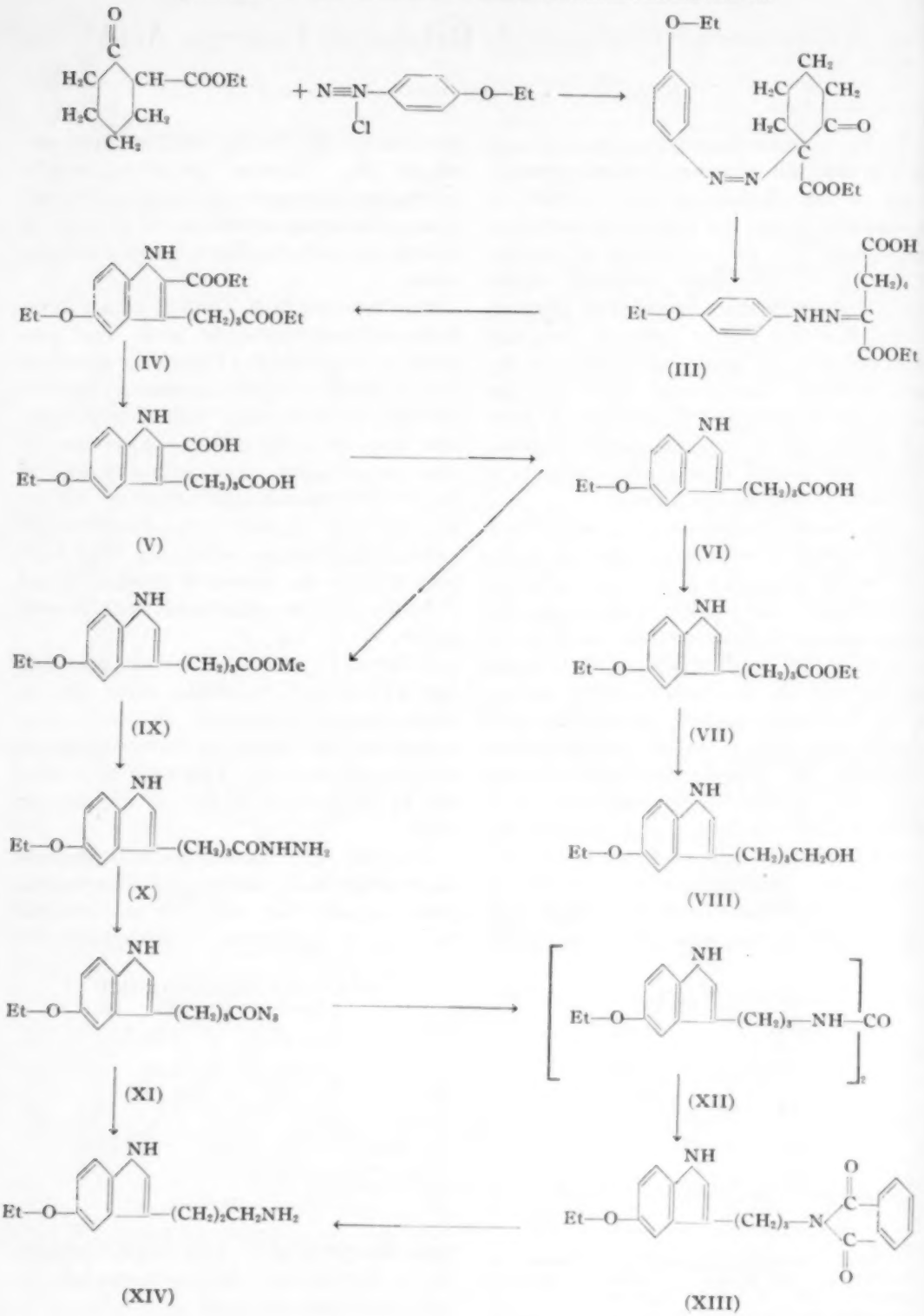
(II)

The (1-ethyl ester of 2-keto-1,7-heptanedioic acid) *p*-ethoxyphenylhydrazone (III) was prepared by application of the Japp-Klingemann reaction using diazotized *p*-

* Based upon a thesis submitted by Hubert W. Murphy to the faculty of Purdue University, Lafayette, Ind., in partial fulfillment of the requirements for the degree of Doctor of Philosophy, December, 1942.

† Eli Lilly and Company Research Fellow, 1941–1942.

‡ The authors wish to express their sincere appreciation to Eli Lilly and Company who sponsored the fellowship that made this work possible.



phenetidine and ethyl 2-keto-1-cyclohexanecarboxylate. This hydrazone was converted to the diethyl ester of 4-(2-carboxy-5-ethoxy-3-indolyl)butanoic acid (IV) from which the dicarboxylic acid (V) was obtained by saponification with alcoholic

sodium hydroxide and subsequent acidification. Attempts to convert the dicarboxylic acid to the dichloride by treatment with thionyl chloride alone or in the presence of pyridine were unsuccessful. Ammonolysis of the diethyl ester did not prove satisfactory for the preparation of the diamide which, it was hoped, could be subjected to the Hofmann degradation and the diamine obtained cyclized to produce 1,2,3,4-tetrahydro-6-ethoxy-9-pyrid(2.3-b)indole.

4-(5-Ethoxy-3-indolyl)butanoic acid (VI) was obtained by the decarboxylization of 4-(2-carboxy-5-ethoxy-3-indolyl)butanoic acid and its ethyl and methyl esters have been prepared. The ethyl ester (VII) was reduced with sodium and dry ethanol to produce 4-(5-ethoxy-3-indolyl)-1-butanol (VIII). 4-(5-Ethoxy-3-indolyl)butanoyl chloride could not be obtained by treating the purified acid with thionyl chloride alone or in the presence of pyridine. Although our results must not be regarded as proving that the acid chlorides of the carbazole and indole derivatives studied cannot be prepared by means of thionyl chloride, all experiments were so decidedly disappointing that it is believed that this method may never prove satisfactory. The treatment is evidently complicated by unknown side reactions which produce extensive resinification in the reaction mixture.

Methyl 4-(5-ethoxy-3-indolyl)butanoate (IX) was treated with an excess of hydrazine hydrate to obtain 4-(5-ethoxy-3-indolyl)butanoyl hydrazide (X). The hydrazide was converted to the corresponding azide (XI) by treatment with nitrous acid. Whenever the azide was added in small portions to boiling distilled water and the reaction mixture worked up, N,N′-bis(3-5-ethoxy-3-indolyl)propyl-urea (XII) could not be crystallized. In order to determine whether the oily product obtained was the desired urea, it was treated with phthalic anhydride according to the method of Manske (8). 3-(5-Ethoxy-3-indolyl)propylphthalimide (XIII) was not isolated from the reaction mixture. The tarry product obtained was treated with hydrazine hydrate according to the method of Ing and Manske (9). Although a considerable amount of phthalylhydrazide was obtained, 3-(5-ethoxy-3-indolyl)propylamine (XIV) could not be isolated, but a cherry-red dye of unknown composition was obtained. The course of the latter reactions will have to be studied further before a satisfactory explanation can be offered.

3(5-Ethoxy-3-indolyl)propylammonium chloride was obtained, in small yield, from the waste liquor produced in the attempted preparation of the urea.

EXPERIMENTAL PART

PART A. INDOLYLCARBOXYLIC ACIDS

1. Attempted Synthesis of 2-Methyl-5-indolylcarboxylic Acid; Preparation of p-Hydrazinobenzoic Acid.—*p*-Aminobenzoic acid was diazotized and reduced according to the method of Veibel and Hauge (10). The yield was 74.5% of the theoretical of *p*-hydrazinobenzoic acid hydrochloride; white crystals, m. p. 248° C. (decompn.).

Acetone (p-Carboxyphenyl)hydrazone.—This hydrazone was easily prepared by adding a slight excess of acetone to a 10% aqueous solution of *p*-hydrazinobenzoic acid hydrochloride. The crystalline product was collected at the pump and purified by recrystallization from 50% alcohol with the aid of activated charcoal; m. p. 230° C. (decompn.).

2-Methylindole.—This compound was isolated whenever 30 Gm. of acetone (*p*-carboxyphenyl)-hydrazone was triturated with 50 Gm. of powdered anhydrous zinc chloride and the mixture heated on an oil bath at 175–185° C., until the reaction mass darkened appreciably. The reaction mixture was made acidic with 5% hydrochloric acid, and the product was collected by distillation with steam. The white, crystalline precipitate was separated from the chilled distillate, dried in a vacuum desiccator over anhydrous calcium chloride, and then distilled (b. p. 272° C./750 mm.); yield 25% of the theoretical. The product was recrystallized from hot water as white needles which, when dried, melted at 59° C. Mixed melting point with a sample of 2-methylindole, 59° C.

Anal. Calcd. for C_9H_9N: N, 10.68%. Found: N, 10.70%.

2. Attempted Synthesis of 3-Ethyl-5-indolylcarboxylic Acid; Preparation of n-Butanal (p-Carboxyphenyl)hydrazone.—This hydrazone was prepared from *n*-butanal and a solution of *p*-hydrazinobenzoic acid hydrochloride by the same procedure used for the preparation of acetone (*p*-carboxyphenyl)hydrazone, except that it was necessary to stir the reaction mixture mechanically. The hydrazone was collected at the pump and purified by recrystallization from 50% alcohol with the aid of activated charcoal. Glistening, slightly yellow platelets which, when dried, melted at 170° C.

Anal. Calcd. for $C_{11}H_{14}N_2O_2$: N, 13.59%. Found: N, 13.63%.

No indole derivative could be isolated in the attempted cyclization of *n*-butanal (*p*-carboxyphenyl)-hydrazone by various modifications of the Fischer method; however, a pronounced fecal odor was usually produced.

PART B. 1,2,3,4-TETRAHYDROCARBAZOLYLCARBOXYLIC ACIDS AND THEIR β-DIETHYLAMINOETHYL ESTERS

1. Synthesis of β-Diethylaminoethyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride; Preparation of Cyclohexanone (p-Carboxyphenyl)hydrazone.—The preparation of this compound from cyclohexanone and *p*-hydrazinobenzoic acid has been described by Collar and Plant (5). The crystals of the hydrazone were collected at the pump, washed with water and dried in a vacuum desiccator over anhydrous calcium chloride. The dried hydrazone was purified by recrystallization from glacial acetic acid with the aid of activated charcoal and obtained as yellow prisms, m. p. 236° C. (decompn.).

1,2,3,4-Tetrahydro-6-carbazolylcarboxylic Acid.—This acid was prepared according to the method reported in the literature by cyclizing cyclohexanone (*p*-carboxyphenyl)hydrazone (5). The acid was purified by recrystallization from glacial acetic acid with the aid of activated charcoal; white prisms, m. p. 281° C.

Sodium 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate.—Twenty-one and one-half grams of the purified acid was added to a solution of 4 Gm. of sodium hydroxide dissolved in 100 cc. of distilled water. The mixture was heated on a steam bath to effect solution, filtered and evaporated to dryness first on a steam bath and then in an oven at 80° C.

β-Diethylaminoethanol.—This substance was prepared by the method described in "Organic Synthesis" (11), b. p. 55° C./100 mm.

(2-Chloroethyl)diethylammonium Chloride.—The procedure of Brit. Pat. 167,781, as described by Gough and King (12), was utilized for the preparation of this compound, m. p. 211° C.

Preparation of β-Diethylaminoethyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride (I).—Twenty grams (1 mole) of the dried finely powdered sodium salt was intimately triturated with 14.5 Gm. (1 mole) of dry (2-chloroethyl)diethylammonium chloride. The mixture was quickly transferred to a reaction flask and heated on an oil bath at 150° C. 1½ hrs. (7). The cooled reaction mixture was dissolved in water and the free ester was extracted with ether from the liquid mixture which had been rendered basic with stronger ammonia water (U. S. P. XI). The ethereal extracts were combined and dried with anhydrous sodium sulfate, filtered, and dry hydrogen chloride was passed into the ethereal filtrate.

The short, fine white needles of the hydrochloride began to separate immediately. The product was collected by filtration at the pump and purified by recrystallization from hot absolute alcohol with the aid of activated charcoal. The yield was 20 Gm., 68% of the theoretical calculated on the sodium salt. The crystalline hydrochloride which had been dried in a vacuum desiccator over anhydrous calcium chloride and solid sodium hydroxide melted at 241–242° C.

Anal. Calcd. for $C_{19}H_{27}ClN_2O_2$: N, 7.99%. Found: N, 7.94%.

2. Attempted Synthesis of 1,2,3,4-Tetrahydro-5- and 1,2,3,4-Tetrahydro-7-carbazolylcarbonyl Chloride; Preparation of m-Hydrazinobenzoic Acid.—Benzoic acid was esterified by using an excess of absolute methanol with hydrogen chloride as a catalyst. The purified ester (b. p. 198° C.) was converted to methyl *m*-nitrobenzoate according to the method described in "Organic Synthesis" (13).

m-Nitrobenzoic acid was obtained by saponification of the ester (14) as light cream-colored crystals, m. p. 140° C. This was reduced to *m*-aminobenzoic acid by treatment with ammonia and hydrogen sulfide; yield 81%, m. p. 173° C.

m-Aminobenzoic acid was then converted to *m*-hydrazinobenzoic acid by the method utilized for the preparation of the *para* isomer.

Cyclohexanone (m-Carboxyphenyl)hydrazone.—This hydrazone was prepared by the method of Collar and Plant (5). It was recrystallized from 50% alcohol as orange prisms which, when dried, melted at 175° C. (decompn.).

1,2,3,4-Tetrahydro-5- and 1,2,3,4-Tetrahydro-7-carbazolylcarboxylic Acid.—These were prepared by cyclization of the hydrazone with 20% sulfuric acid. The acids were separated by fractional crystallization from glacial acetic acid and purified by recrystallization of their esters (5). Neither acid (m. p. 286° C. and m. p. 212° C.) could be converted to the acid chloride by treatment with thionyl chloride alone or in the presence of pyridine.

Since no proof of the structures of the acids was obtained, no attempt was made to prepare the β-diethylaminoethyl esters.

3. Synthesis of β-Diethylaminoethyl 1,2,3,4-Tetrahydro-8-carbazolylcarboxylate Hydrochloride; Preparation of o-Hydrazinobenzoic Acid.—The hydrochloride of this acid was prepared by the procedure used for the preparation of the *para* isomer starting with 27.4 Gm. of anthranilic acid. The following modification was found necessary to remove the *o*-diazobenzimide which formed. After treatment with zinc powder and glacial acetic acid, the mixture was filtered and 20 cc. of concentrated hydrochloric acid was added. The acidified filtrate was allowed to stand for 24 hrs. and the mixture was filtered to remove the imide. Only 500 cc. of concentrated hydrochloric acid was added to the filtrate to effect precipitation of the *o*-hydrazinobenzoic acid hydrochloride. The product was purified by recrystallization from water with the aid of activated charcoal.

Cyclohexanone (o-Carboxyphenyl)hydrazone.—A warm aqueous solution of *o*-hydrazinobenzoic acid hydrochloride was stirred with an excess of cyclohexanone. The crystals of the hydrazone were col-

lected by filtration at the pump, washed, dried and recrystallized from benzene; pale yellow needles, m. p. 162° C.

1,2,3,4-Tetrahydro-8-carbazolylcarboxylic Acid.—Cyclization of the hydrazone, described above, with aqueous sulfuric acid produced the desired acid (5). The acid was collected by filtration, washed, dried and recrystallized from benzene as colorless prisms, m. p. 203° C. Ethyl ester, m. p. 76° C.

Sodium 1,2,3,4-Tetrahydro-8-carbazolylcarboxylate.—Ten and three-quarters grams of the recrystallized acid was added to a solution of 2 Gm. of sodium hydroxide in 50 cc. of distilled water. The mixture was heated to effect solution, filtered, and the filtrate was evaporated on a water bath. The resi[illegible] yellow crystalline mass was then dried for several days in an oven at 80° C.

Preparation of β-Diethylaminoethyl 1,2,3,4-Tetrahydro-8-carbazolylcarboxylate Hydrochloride (II).—Ten grams of the dried, finely powdered sodium salt was intimately triturated with 7.25 Gm. of (2-chloroethyl)diethylammonium chloride and the mixture was transferred to a flask and heated on an oil bath at 150° C. for 2 hrs. The cooled reaction mixture was treated with 250 cc. of hot distilled water and the liquid was filtered. The filtrate was made alkaline with 20 cc. of stronger ammonia water, and the free ester was collected from the cold mixture by filtration. The dried ester was dissolved in benzene, and dry hydrogen chloride was passed into the solution. The crystals of the ester hydrochloride were collected by filtration at the pump, washed with a little ether and recrystallized from absolute ethanol with the aid of activated charcoal; white platelets, stable in air, m. p. 208° C.

Anal. Calcd. for $C_{19}H_{27}ClN_2O_2$: N, 7.99%. Found: N, 7.96%.

4. Attempted Synthesis of 1,2,3,4-Tetrahydro-4-chloro-6-carbazolylcarboxylic Acid; Preparation of 2-Chloro-1-cyclohexanone.—This ketone was obtained from cyclohexanol using the method of Meyer (15) b. p. 73.5–74.5° C./7 mm. (Uncor.); $n^{D}_{20°}$ = 1.4824 (Literature, $n^{D}_{20°}$ 1.4825).

A satisfactory method for the preparation of the (*p*-carboxyphenyl)- or the (*o*-carboxyphenyl)hydrazone was not developed.

(2-Keto-1-cyclohexyl)diethylamine.—This substance was prepared by treating 2-chlorocyclohexanone with an excess of diethylamine in the presence of dry sodium iodide at room temperature. It was isolated by the general method employed for a basic substance and finally distilled at 119–121° C./22 mm. (Uncor.) as a light yellow oil with a strong amine odor. The compound turned brown and solidified after standing for several weeks; b. p. 188° C./742 mm.; $n^{D}_{20°}$ = 1.4929.

Anal. Calcd. for $C_{10}H_{19}NO$: N, 8.27%. Found: N, 8.36%.

The hydrochloride was obtained as extremely deliquescent, white prisms, m. p. 226–228° C.

5. Attempted Synthesis of 1,2,3,4-Tetrahydro-1-methyl-1,4-(dimethylmethylene)-6-carbazolylcarboxylic Acid; Preparation of Camphor (p-Carboxyphenyl)-hydrazone.—Camphor was dissolved in alcohol, and this solution was added to an excess of *p*-hydrazinobenzoic acid hydrochloride and sodium acetate. The mixture was refluxed for 2 hrs., and then most of the alcohol was removed at reduced pressure. The residue was poured into cold water and, after standing, the crystalline mass was collected by filtration. The hydrazone was recrystallized from 75% alcohol with the aid of activated charcoal; short white needles, m. p. 251° C. The yield was practically quantitative.

Anal. Calcd. for $C_{17}H_{22}N_2O_2$; N, 9.75%. Found: N, 9.89%.

All attempts to produce cyclization were unsuccessful. Acid catalysts in the presence of alcohol produced camphor (*p*-carbethoxyphenyl)hydrazone which was purified by recrystallization from 75% alcohol; small white prisms, m. p. 180–181° C.

Anal. Calcd. for $C_{19}H_{26}N_2O_2$: N, 8.91%. Found: N, 8.86%.

Aqueous acid catalysts effected hydrolysis of the hydrazone.

6. Attempted Synthesis of 1,2,3,4-Tetrahydro-1-methyl-1,4-(dimethylmethylene)-8-carbazolylcarboxylic Acid; Preparation of Camphor (o-Carboxyphenyl)-hydrazone.—This hydrazone was obtained by the method described for the *para* isomer. It was recrystallized from glacial acetic acid as stout yellow needles, m. p. 224–226° C. (decompn.).

Anal. Calcd. for $C_{17}H_{22}N_2O_2$: N, 9.75%. Found: N, 9.59%.

Cyclization by the Fischer method could not be effected.

PART C. 4-(2-CARBOXY-5-ETHOXY-3-INDOLYL)-BUTANOIC ACID AND DERIVED PRODUCTS

1. Synthesis of 4-(2-Carboxy-5-ethoxy-3-indolyl)-butanoic Acid; Preparation of (1-Ethyl Ester of 2-Keto-1,7-heptanedioic Acid) p-Ethoxyphenylhydrazone (III).—Diethyl oxalate was prepared by the esterification of anhydrous oxalic acid (16) with sulfuric acid as a catalyst. Ethyl 2-keto-1-cyclohexanecarboxylate was prepared from diethyl oxalate and cyclohexanone as described by Kötz (17).

p-Phenetidine was prepared from acetophenetidin (U. S. P. XI) by deacetylization (18).

The hydrazone was prepared by application of the Japp-Klingemann reaction as described by Jackson and Manske (19) to diazotized *p*-phenetidine and the cyclic ester. The hydrazone separated from the acidified reaction mixture as a viscous, dark reddish brown oil in which, after it had been washed by decantation, crystallization was initiated by stirring with a glass rod. The crude hydrazone was purified by recrystallization from benzene with the aid of activated charcoal; lemon-yellow prisms, m. p. 114° C.

Anal. Calcd. for $C_{17}H_{24}N_2O_5$: N, 8.33%. Found: N, 8.24%.

Preparation of the Diethyl Ester of 4-(2-Carboxy-5-ethoxy-3-indolyl)butanoic Acid (IV).—The crude hydrazone was added to 10% alcoholic sulfuric acid and the mixture was refluxed for 2½ hrs. Most of the alcohol was distilled off at 23 mm., and the residue was dissolved in a mixture of benzene and ether (1:2). The mixture was washed with water and sodium bicarbonate solution and then dried with anhydrous sodium sulfate.

The solvents were distilled off under reduced pressure, and the residue was treated with hot 50% alcohol. The crystals were collected by filtration from the cold supernatant liquid. The sludge remaining in the bottom of the flask was reheated with the same solvent, and a further crop of crystals was obtained.

The air-dried crystals were purified by recrystallization from 50% aqueous acetone. The dried, faintly buff-colored, glistening flattened needles melted at 93° C. The yield was 34.3% of the theoretical calculated from the cyclic ester used for the preparation of the hydrazone.

Anal. Calcd. for $C_{19}H_{25}NO_5$: N, 4.03%. Found: N, 4.04%.

Preparation of 4-(2-Carboxy-5-ethoxy-3-indolyl)-butanoic Acid (V).—The purified diethyl ester was saponified with alcoholic sodium hydroxide at room temperature by allowing a mixture to stand for a few days. The precipitated sodium salt was collected by filtration, dissolved in hot distilled water, and the mixture was filtered with the aid of activated charcoal. The free acid was precipitated by adding an excess of hydrochloric acid to the cold filtrate. The acid was collected by filtration and purified by several recrystallizations from 50% acetic acid. It was dried in a vacuum desiccator over anhydrous calcium chloride and sodium hydroxide pellets and obtained as a fine white, microcrystalline powder which melted at 206° C.

Anal. Calcd. for $C_{15}H_{17}NO_5$: N, 4.81%. Found: N, 4.72%.

2. Synthesis of 4-(5-Ethoxy-3-indolyl)butanoic Acid and Derivatives; Preparation of 4-(5-Ethoxy-3-indolyl)butanoic Acid (VI).—4-(2-Carboxy-5-ethoxy-3-indolyl)butanoic acid was decarboxylated by heating it with a small amount of powdered glass on an oil bath at 220–230° C. The cooled reaction mixture was treated with hot distilled water, the mixture was filtered, and the acid which precipitated from the cold filtrate was collected and purified by recrystallization from distilled water; white needles, m. p. 133° C.; yield 70% of the theoretical.

Anal. Calcd. for $C_{14}H_{17}NO_3$: N, 6.17%. Found: N, 6.06%.

Ethyl 4-(5-Ethoxy-3-indolyl)butanoate (VII).—The purified acid was dissolved in absolute ethanol and esterified by refluxing the mixture in the presence of dry hydrogen chloride for 2 hrs.

Most of the excess of alcohol was removed under reduced pressure, the residue was dissolved in ether, and the ethereal liquid was washed with water and sodium bicarbonate solution. The ester was obtained by evaporating the ether and then recrystallized from 40% alcohol with the aid of activated charcoal. It was dried in a vacuum desiccator over anhydrous calcium chloride and sodium hydroxide pellets and obtained as white needles which melted at 69° C.

Anal. Calcd. for $C_{16}H_{21}NO_3$: N, 5.09%. Found: N, 5.15%.

Methyl 4(5-Ethoxy-3-indolyl)butanoate (IX).—This ester was prepared from the acid and absolute methanol by the method described above. It was purified by recrystallization from 50% methanol with the aid of activated charcoal; fine white needles, m. p. 84° C.

Preparation of 4-(5-Ethoxy-3-indolyl)-1-butanol (VIII).—This alcohol was prepared by reducing the dried, purified ethyl ester with sodium and absolutely dry ethanol. It was purified by extracting with ether, washing with water, and drying the ethereal extract. The alcohol was distilled, after removal of the ether, at 215° C./2 mm. Crystallization could not be effected. The colorless oil became dark brown on standing; $n_{20^\circ}^{D} = 1.5680$.

Anal. Calcd. for $C_{14}H_{19}NO_2$: N, 6.01%. Found: N, 6.02%.

4-(5-Ethoxy-3-indolyl)-1-butyl Phenylurethan.—This derivative was prepared by the general method from the anhydrous alcohol. It was recrystallized from carbon tetrachloride as white needles, m. p. 117–118° C.

Preparation of 4-(5-Ethoxy-3-indolyl)butanoyl Hydrazide (X).—Forty-one grams of purified methyl 4-(5-ethoxy-3-indolyl)butanoate, 37 Gm. of 40% hydrazine hydrate and 45 cc. of ethanol were placed in an acetylization flask. The mixture was heated under reflux for an hour on an oil bath at 130° C. The air-cooled condenser was disconnected and the temperature was raised to 145° C. to effect the removal of most of the water, alcohols and excess of hydrazine hydrate. The cooled residue solidified and the crystalline mass was disintegrated, collected on a Büchner funnel and washed with cold water and then with cold ethanol.

The crude hydrazide was recrystallized from alcohol with the aid of activated charcoal and dried in a vacuum desiccator. The yield was 35 Gm. of fine white prisms, m. p. 157° C.

Anal. Calcd. for $C_{14}H_{19}N_3O_2$: N, 17.41%. Found: N, 17.52%.

Preparation of 4-(5-Ethoxy-3-indolyl)butanoyl Azide (XI).—Thirty grams of dried, purified hydrazide was dissolved in 300 cc. of glacial acetic acid, and the solution was then placed in a salt and ice bath. About 400 Gm. of crushed ice was added to the solution and, when the temperature of the mixture had fallen to 0° C., a solution of 12 Gm. of sodium nitrite in 50 cc. of water was added slowly with mechanical agitation.

An oily product separated first, which after a short time changed to the yellow crystalline azide. The azide was collected by filtration, thoroughly

washed with cold distilled water and then used for the attempted preparation of the urea.

Preparation of 3-(5-Ethoxy-3-indolyl)propylammonium Chloride (XIV).—The waste liquor, obtained from the decomposition of the azide in the attempted preparation of the urea, was treated by the general method for the isolation of a basic substance. After partition between immiscible solvents had been completed, the ethereal extract of the amine was dried by the addition of a few sodium hydroxide pellets. The liquid was filtered, and the ether was removed from the filtrate. The residue was dissolved in 20 cc. of acetone, and 50 cc. of cold ether saturated with hydrogen chloride was added. The white needles of the amine hydrochloride were collected by filtration, washed with cold acetone and dried in a vacuum desiccator over anhydrous calcium chloride and sodium hydroxide. The yield was approximately 0.75 Gm., m. p. 205° C.

Anal. Calcd. for $C_{13}H_{19}ClN_2O$: N, 11.00%. Found: N, 11.03%.

SUMMARY

1. The hydrochlorides of the β-diethylaminoethyl esters of 1,2,3,4-tetrahydro-6- and 1,2,3,4-tetrahydro-8-carbazolylcarboxylic acid and 3-(5-ethoxy-3-indolyl)propylammonium chloride have been prepared for pharmacological investigation.

2. The synthesis of several substituted hydrazones and indole derivatives is described.

3. The production of 2-methylindole by the cyclization and simultaneous decarboxylization of acetone (*p*-carboxyphenyl)-hydrazone is reported.

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Determination of Camphor in Spirit of Camphor

Modification of the United States Pharmacopoeia X Method*

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The tenth decennial revision of the United States Pharmacopœia offers for the determination of camphor in spirit of camphor a method which is based on the rotatory power of camphor.

In a previous communication (1), the rotatory power of camphor in alcoholic solutions was studied. The solutions were made to contain varying amounts of

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camphor (from 1 Gm. to 50 Gm.) in enough alcohol of varying strengths (from 35% by volume to absolute) to make 100 cc. of solution. It was concluded from this study that the specific rotation increases as the concentration of the camphor and the strength of alcohol increase. Because of these variations in the specific rotations, it was thought that the U. S. P. X method of analysis for spirit of camphor could not give accurate results with samples containing amounts of alcohol and camphor differing from those contained in the U. S. P. preparations.

EXPERIMENTAL

Several solutions containing varying amounts of camphor in varying percentages, *v/v*, of alcohol were prepared from high grade camphor that had been carefully purified by at least 1 and sometimes by 2 sublimations. These solutions were then assayed by the U. S. P. X method. The rotatory power of each solution was determined by means of a saccharimeter equipped with a Ventzke scale. Five readings were made on each sample. A sodium vapor lamp furnished the necessary light, but it was noted that white light used in conjunction with a 3-cm. cell containing 3% potassium bichromate solution gave equally accurate results, when the percentage strength of camphor was not excessive. The control test solution was prepared by dissolving 10 Gm. of camphor in sufficient alcohol (95.00%, *v/v*) to make 100 cc. This solution showed a reading of 24.10. All samples assayed were compared with this solution because all were prepared from the same sample of pure sublimed camphor. However, in a previous investigation (1) the authors pointed out that the specific rotation of 8 different samples of camphor (10% camphor in 95% alcohol) varied by only 0.08, a difference which corresponds to a saccharimeter reading of 0.05.

Examination of Table I, showing the results of assay by the U. S. P. X method, indicates that accurate results can be obtained only if the spirit contains exactly 10 Gm. of camphor dissolved in enough alcohol (exactly 95.00%, *v/v*) to make 100 cc. If the sample contains less than 10% camphor, the assay usually gives results that are lower than the actual percentage of camphor present. If the percentage camphor in the sample is above 10%, the assay usually gives varying results.

Furthermore, in this method of assay, if the spirit has been made with alcohol of greater than 95% strength, the results usually will be higher than the theoretical values. On the other hand, if the spirit has been made with alcohol of lower percentage strength than the official U. S. P. alcohol, the results usually are lower than the theoretical values.

Two factors, therefore, cause the variation of results. First, the rotatory power of alcoholic solutions of camphor is not affected proportionally by an increase in the camphor content. Second, the rotatory power of the camphor solutions is influenced by an increasing of the alcoholic content of the solvent. Both of these factors could be overcome by reference to tables of saccharimeter readings or charts, if the strength of the alcohol that had been used in making the original sample was known first.

However, it would seem more logical to construct tables and charts for solutions containing definite percentages of alcohol rather than charts specifying the percentages of alcohol that had been used in the manufacture of the particular sample.

The solutions for this part of the study were made to contain amounts of camphor varying from 1 Gm. to 45 Gm. per 100 cc. and percentages of alcohol varying from 35% to 97.5%, *v/v*. In making each solution, the proper amount of camphor was weighed into a previously dried and weighed 100-cc. volumetric flask, and the required amount of alcohol at 20° C. was run into the flask from a burette. After the camphor was completely dissolved, distilled water was slowly added until the liquid stood nearly at the mark. The flask and its contents were then placed in a water bath at 20° C., and the flask was rotated (not shaken) at frequent intervals. Finally enough distilled water was added to make the product measure 100 cc.

TABLE I.—DETERMINATION OF CAMPHOR BY MEANS OF THE U. S. P. X METHOD

Camphor, %	Alcohol, % Used	Reading of Solution	Camphor, % Determined	Variation, %
5.00	99.50	12.57	5.21	+0.21
5.00	95.00	11.83	4.90	−0.10
5.00	90.11	11.46	4.75	−0.25
5.00	80.28	10.90	4.52	−0.48
5.00	70.31	10.45	4.33	−0.67
5.00	60.28	10.14	4.20	−0.80
7.50	99.50	18.94	7.85	+0.35
7.50	95.00	17.90	7.42	−0.08
7.50	90.11	17.33	7.18	−0.32
7.50	80.28	16.60	6.88	−0.62
7.50	70.31	16.06	6.66	−0.84
7.50	60.28	15.69	6.50	−1.00
10.00	95.00	24.10	10.00	0.00
10.00	95.00	24.08	9.98	−0.02
10.00	95.00	24.20	10.03	+0.03
10.00	99.50	25.38	10.52	+0.52
10.00	99.50	25.46	10.56	+0.56
10.00	90.11	23.28	9.61	−0.39
10.00	80.28	22.34	9.26	−0.74
10.00	70.31	21.70	9.00	−1.00
10.00	60.28	21.44	8.89	−1.11
12.50	99.50	31.89	13.22	+0.72
12.50	95.00	30.20	12.52	+0.02
12.50	90.11	29.30	12.15	−0.35
12.50	80.28	28.25	11.71	−0.79
12.50	70.31	27.56	11.39	−1.11
12.50	60.28	27.23	11.29	−1.21
15.00	99.50	38.41	15.92	+0.92
15.00	95.00	36.53	15.14	+0.14
15.00	90.11	35.44	14.69	−0.31
15.00	80.28	34.20	14.18	−0.82
15.00	70.31	33.53	13.90	−1.10

Five different percentages of alcohol varying from 65% to 99.5% were used in making these solutions. The lowest possible percentage alcohol was used in making each particular solution in order to minimize loss of alcohol by evaporation, to minimize precipitation of camphor by the addition of water, and to minimize the generation of heat upon the addition of water. Table II indicates how the various percentages of alcohols were used in preparing the solutions. The figures in column 4 were obtained by dividing the cubic centimeters of absolute alcohol desired in the preparation by the percentage of alcohol being used.

In order to show that this method of making solutions was sound, several dilutions of alcohols were prepared from these standard alcohols, and the percentage strengths of the dilutions were determined in the usual way by means of specific gravity and densimetric tables. The determined percentages of alcohol from the analyses are given in column 4 of Table III. In no case was the variation from the theoretical greater than 0.07%.

TABLE II.—EXAMPLES ILLUSTRATING HOW SOLUTIONS WERE MADE FROM THE VARIOUS ALCOHOLS

Camphor, Gm.	Alcohol, % Desired	Alcohol, % Used	Alcohol, Cc. Used
1.0	97.5	99.50	97.99
22.5	75.0	99.50	75.38
10.0	85.0	95.26	89.23
5.0	80.0	85.15	93.95
10.0	65.0	75.28	86.34
10.0	55.0	65.00	84.61

TABLE III.—PRODUCTION OF DILUTED ALCOHOLS FROM THE VARIOUS PERCENTAGE ALCOHOLS

Alcohol, % Used	Alcohol, Cc. Used	Theoretical % after Dilution	Determined % after Dilution	Variation, %
99.50	95.48	95	94.93	−0.07
99.50	95.48	95	95.00	0.00
95.26	94.48	90	90.00	0.00
85.15	93.95	80	79.95	−0.05
75.28	92.99	70	70.03	+0.03
65.00	92.31	60	59.99	−0.01
95.26	62.98	60	60.03	+0.03
95.26	62.98	60	59.98	−0.02

The camphor solutions, prepared as described, were read at 20° C. in a saccharimeter with a Ventzke scale. Monochromatic light from a sodium vapor lamp was used. The polariscope tubes used in this work were 2 dm. in length for all solutions except the 40% and 45% camphor solutions. For these solutions a 1-dm. tube was used, and the readings were doubled before being recorded in the tables.

Ten readings were taken for each solution, and the average reading was computed. The zero point was determined frequently and the necessary corrections were made. These average readings are tabulated in Table IV. The readings show to a certain extent how the rotatory powers are affected by increased camphor content and increased alcohol content; but the specific rotations show decidedly how the rotatory powers are affected.

From these readings the specific rotations were computed by means of the formula

$$\frac{100a}{2.8835 \times lc} = \text{specific rotation}$$

in which a is the saccharimeter reading, l is the length of the tube in decimeters, and c is the concentration of camphor in Gm./100 cc. of solution. The specific rotations are tabulated in Table V. The lowest specific rotation (27.56) is for 1% camphor and 35% alcohol; whereas the highest figure (46.67) is for 45% camphor and 50% alcohol—a difference in specific rotation of 19.11.

The specific rotations, percentages alcohol and percentages camphor form a very interesting set of charts. In Figs. 1 and 2 the specific rotations are represented by the ordinates and the concentrations of camphor are represented by the abscissas. Each curve represents a single percentage of alcoholic contents. It will be noticed that the curves rise rapidly at first, show a tendency to flatten out later and finally rise rapidly again.

In Figs. 3 and 4 the specific rotations are again represented by the ordinates, but the percentages of

TABLE IV.—SACCHARIMETER READINGS OF SOLUTIONS CONTAINING VARIOUS PERCENTAGES OF CAMPHOR (w/v) AND VARIOUS PERCENTAGES OF ALCOHOL (v/v)

Camphor, %	Alcohol, %													
	35	40	45	50	55	60	65	70	75	80	85	90	95	97.5
1.0	1.59	1.62	1.66	1.70	1.75	1.80	1.85	1.92	1.98	2.06	2.14	2.24	2.35	2.44
2.5	..	..	4.64	4.70	4.79	4.89	4.99	5.11	5.25	5.40	5.57	5.78	6.07	
5.0	..	..	..	10.06	10.16	10.28	10.46	10.67	10.91	11.18	11.50	11.89		
7.5	..	..	..	15.65	15.77	15.93	16.17	16.45	16.67	17.14	17.60	18.34		
10.0	..	..	..	....	21.51	21.68	21.93	22.29	22.72	23.29	24.10			
12.5	..	..	..	....	27.38	27.59	27.90	28.34	28.91	29.64	30.95			
15.0	..	..	..	....	33.37	33.62	34.03	34.59	35.34	36.48				
17.5	..	..	..	....	39.48	39.74	40.25	41.02	42.09	44.05				
20.0	..	..	..	....	45.72	46.06	46.79	47.80	49.29					
22.5	..	..	..	....	52.19	52.69	53.64	55.06	57.71					
25.0	..	..	..	....	58.90	59.59	60.87	62.82						
30.0	..	..	..	....	72.87	74.45	76.97							
35.0	..	..	..	86.47	88.37	91.29								
40.0	..	..	..	102.54	106.06									
45.0	..	..	..	121.12										

TABLE V.—SPECIFIC ROTATIONS OF SOLUTIONS CONTAINING VARIOUS PERCENTAGES OF CAMPHOR (*w/v*) AND VARIOUS PERCENTAGES OF ALCOHOL (*v/v*)

Camphor, %	Alcohol, %													
	35	40	45	50	55	60	65	70	75	80	85	90	95	97.5
1.0	27.56	28.14	28.79	29.47	30.26	31.13	32.11	33.20	34.38	35.70	37.15	38.79	40.78	42.23
2.5	...	...	32.16	32.63	33.23	33.89	34.62	35.45	36.40	37.45	38.64	40.07	42.10	
5.0	...	...	...	34.88	35.23	35.67	36.27	37.00	37.84	38.77	39.88	41.21		
7.5	...	...	...	36.19	36.45	36.83	37.38	38.02	38.77	39.62	40.70	42.39		
10.0	...	...	...	...	37.30	37.59	38.02	38.64	39.40	40.38	41.70			
12.5	...	...	...	...	37.98	38.28	38.70	39.32	40.10	41.12	42.94			
15.0	...	...	...	...	38.58	38.87	39.34	39.99	40.86	42.17				
17.5	...	...	...	...	39.12	39.38	39.88	40.65	41.71	43.65				
20.0	...	...	...	...	39.64	39.94	40.57	41.45	42.74					
22.5	...	...	...	...	40.22	40.61	41.35	42.43	44.40					
25.0	...	...	...	...	40.85	41.33	42.22	43.57						
30.0	...	...	...	...	42.12	43.03	44.49							
35.0	...	...	...	42.84	43.78	45.23								
40.0	...	...	...	44.45	45.98									
45.0	...	...	...	46.67										

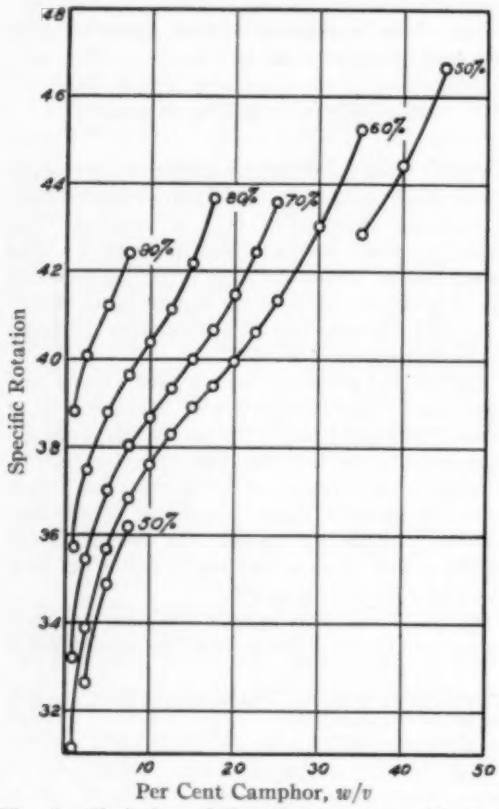

Fig. 1.—Variation of Specific Rotation with Percentage Camphor.

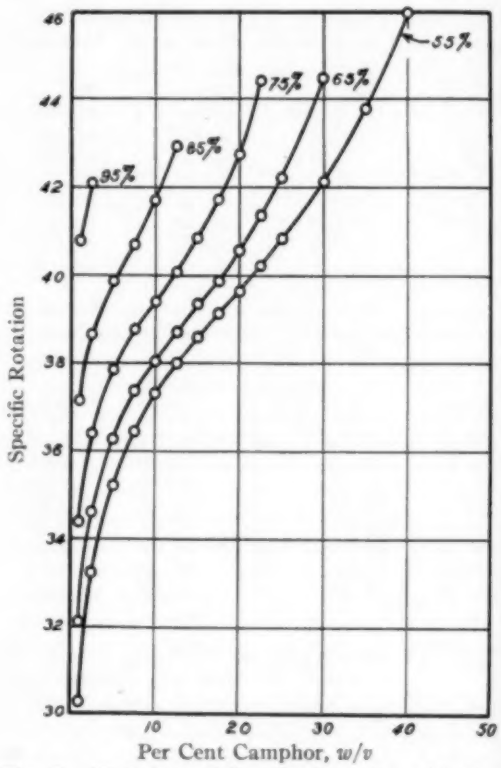

Fig. 2.—Variation of Specific Rotation with Percentage Camphor.

alcohol in the solution are represented by the abscissas. Each curve represents a single percentage strength of camphor. The curves for the lower concentrations of camphor tend to show a greater rate of ascent than those for the higher concentrations.

In Fig. 5 the ordinates represent the Ventzke scale readings, and the abscissas represent the strength of alcohol. Each curve represents a single percentage strength of camphor. Only a few of the most important points are shown in the plotting of the curves in this chart.

It has been stated that the alcohol content of the spirit must be known before the modified method for the determination of camphor can be used. A study of several methods for the determination of the alcohol content of spirit of camphor was made. The results of the study of the alcohol determination

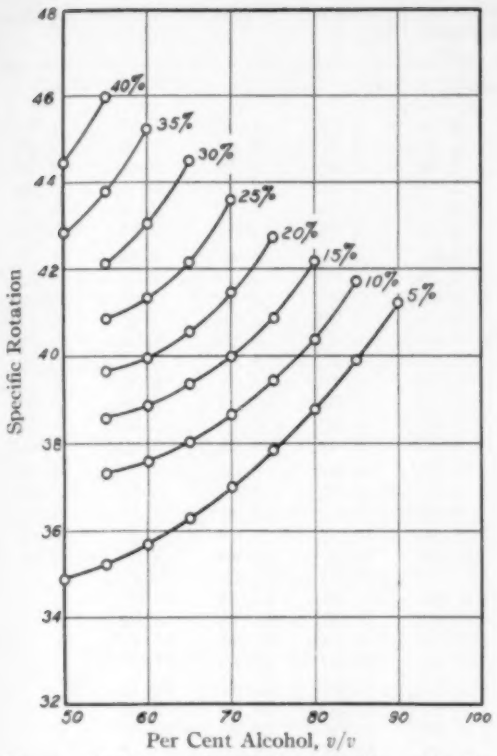

Fig. 3.—Variation of Specific Rotation with Percentage Alcohol.

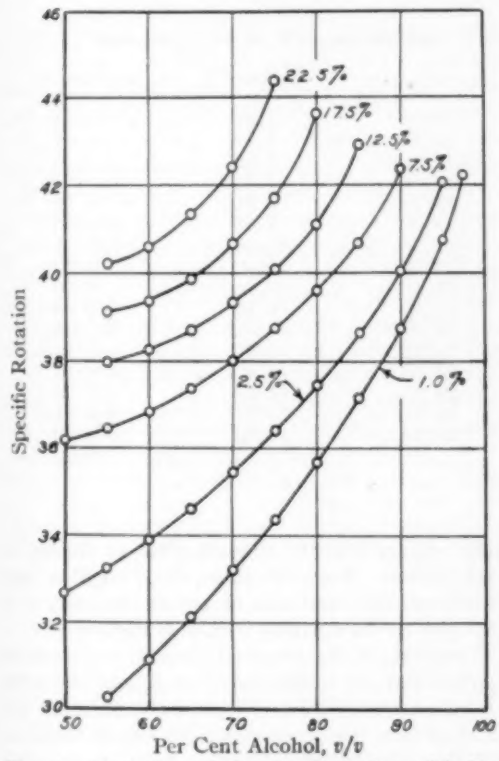

Fig. 4.—Variation of Specific Rotation with Percentage Alcohol.

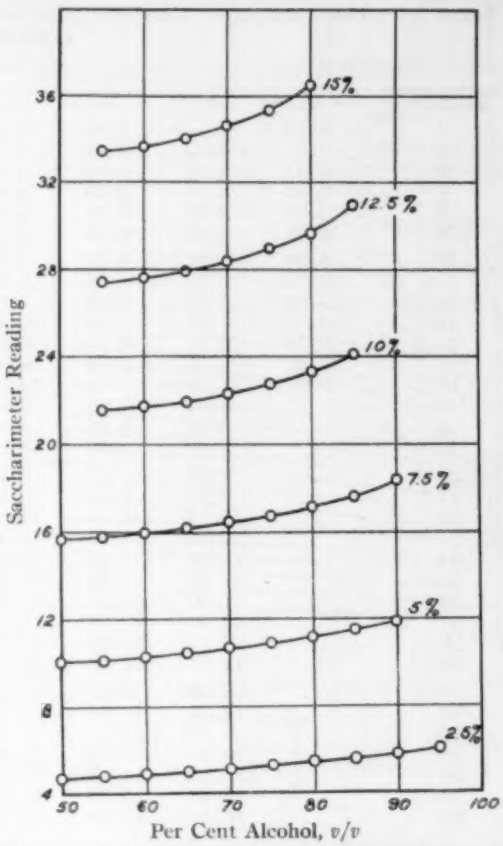

Fig. 5.—Variation of Saccharimeter Reading with Percentage Alcohol.

methods will be reported in a future communication.

It would be quite unsatisfactory to use any of the charts or tables given thus far for calculating the amount of camphor from the optical activity of the solutions and the alcohol determinations since specific rotations were used in the first four charts. In Fig. 5, where saccharimeter readings are involved, the curves are widely separated, and interpolation would be difficult. Table IV could not be used successfully, because it has been shown that interpolation is not of a linear type. By means of Table IV and Fig. 6, a new type of tables and charts was constructed. In Fig. 6 the saccharimeter readings as the ordinates are plotted against the percentages of camphor as the abscissas. Each curve represents a definite percentage of alcohol for all the solutions involved.

In order to make use of the saccharimeter readings in the analysis of camphor, a new chart was constructed with the percentages of camphor as ordinates and the percentages of alcohol as the abscissas. The chart contains a series of curves, each one representing some definite whole-numbered saccharimeter reading. In preparing the data, it was necessary to find several combinations of camphor and alcohol, each of which would possess this whole-numbered saccharimeter reading. This condition was accomplished by means of Fig. 6, by locating the

TABLE VI.—PERCENTAGES OF CAMPHOR (*w/v*) IN SOLUTIONS POSSESSING WHOLE-NUMBERED SACCHARIMETER READINGS

Saccharimeter Reading	Alcohol, % (*v/v*)								
	50	55	60	65	70	75	80	85	90
4	2.15	2.11	2.05	2.02	1.98	1.92	1.87	1.81	1.74
5	2.67	2.62	2.54	2.51	2.45	2.38	2.32	2.24	2.15
6	3.21	3.14	3.05	3.02	2.94	2.86	2.78	2.69	2.59
7	3.61	3.56	3.49	3.44	3.36	3.28	3.19	3.10	2.98
8	3.98	3.94	3.89	3.82	3.75	3.66	3.57	3.47	3.35
9	4.48	4.43	4.38	4.31	4.22	4.13	4.02	3.91	3.78
10	4.98	4.93	4.87	4.79	4.69	4.59	4.47	4.35	4.20
11	5.44	5.39	5.31	5.24	5.16	5.03	4.93	4.77	4.62
12	5.89	5.81	5.74	5.67	5.58	5.46	5.36	5.21	5.02
13	6.33	6.27	6.20	6.11	6.01	5.90	5.78	5.61	5.42
14	6.78	6.71	6.65	6.54	6.44	6.33	6.20	6.02	5.81
15	7.21	7.15	7.07	6.97	6.85	6.75	6.60	6.42	6.19
16	7.65	7.59	7.51	7.39	7.29	7.16	7.01	6.83	6.57
17	..	8.04	7.95	7.85	7.72	7.58	7.42	7.24	6.96
18	..	8.46	8.39	8.29	8.19	8.02	7.83	7.62	7.34
19	..	8.90	8.83	8.72	8.57	8.44	8.24	8.02	7.70
20	..	9.35	9.27	9.15	9.03	8.87	8.66	8.41	8.07
21	..	9.78	9.71	9.59	9.46	9.30	9.08	8.81	8.46
22	..	10.23	10.15	10.04	9.90	9.73	9.49	9.18	8.81
23	..	10.65	10.55	10.45	10.30	10.13	9.88	9.55	9.17
24	..	11.06	10.96	10.85	10.68	10.53	10.26	9.96	9.55
25	..	11.48	11.40	11.27	11.10	10.93	10.66	10.31	
26	..	11.90	11.81	11.69	11.51	11.33	11.05	10.67	
27	..	12.33	12.24	12.11	11.91	11.73	11.45	11.04	
28	..	12.75	12.66	12.53	12.33	12.13	11.84	11.41	
29	..	13.17	13.06	12.93	12.74	12.52	12.25	11.77	
30	..	13.59	13.49	13.34	13.15	12.91	12.62	12.13	
31	..	14.00	13.90	13.75	13.55	13.30	13.00	12.50	
32	..	14.44	14.32	14.16	13.96	13.68	13.35	12.84	
33	..	14.85	14.74	14.56	14.36	14.07	13.70	13.16	
34	..	15.25	15.15	14.99	14.75	14.46	14.07	13.49	
35	..	15.65	15.55	15.39	15.15	14.85	14.45	13.85	
36	..	16.07	15.99	15.80	15.54	15.24	14.80	14.20	

TABLE VII.—ANALYSIS OF SPIRIT OF CAMPHOR BY THE MODIFIED U. S. P. X METHOD

Camphor, %	Alcohol, %	Alcohol, % Determined	Saccharimeter Reading	Camphor, % Determined	Variation, % Camphor
5.0	55.00	54.72	10.06	4.97	−0.03
5.0	65.00	64.76	10.30	4.91	−0.09
5.0	90.00	89.80	12.06	5.08	+0.08
7.5	70.00	69.72	16.56	7.53	+0.03
7.5	87.93	87.64	18.10	7.50	0.00
7.5	90.00	89.68	18.30	7.45	−0.05
10.0	70.00	69.64	22.25	10.01	+0.01
10.0	80.00	79.72	23.25	10.00	0.00
10.0	85.31	85.04	24.10	10.00	0.00
10.0	60.00	59.68	21.53	9.92	−0.08
10.0	85.31	85.00	24.17	10.02	+0.02
12.5	65.00	64.76	27.62	12.35	−0.15
12.5	75.00	74.80	28.75	12.40	−0.10
12.5	80.00	79.76	29.85	12.58	+0.08
15.0	65.00	64.72	34.01	15.01	+0.01
15.0	80.00	79.68	36.24	14.88	−0.12
				Av.	−0.02

points, and by interpolation of the readings given in Table IV. Inasmuch as this was not purely a linear type of interpolation, the chart was used for the higher readings and the tables for the lower ones because of their close association on the chart.

As a result of this study, Table VI could be prepared. In this table the saccharimeter readings are given at the extreme left, and the amounts of camphor are given to the right in Gm./100 cc. of solution necessary to produce that reading when the alcoholic content is of the strength given at the top of each column. From this table, Fig. 7 could be prepared, and this chart may be used in the analysis of the spirit by the modified U. S. P. X method.

Therefore, in the proposed method, the alcoholic content and the saccharimeter reading of the spirit are determined. Next is located the point on the abscissa line that corresponds to this determined value for the alcohol. As an example, suppose this value for alcohol is 67.5%. From this point one

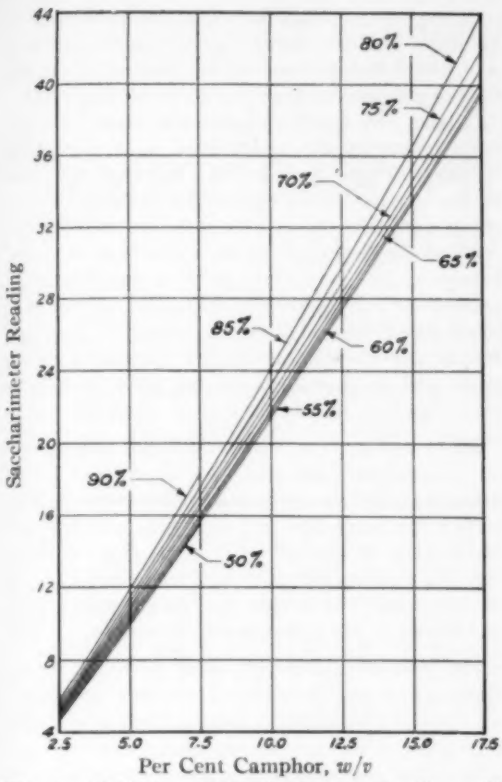

Fig. 6.—Variation of Saccharimeter Reading with Percentage Camphor.

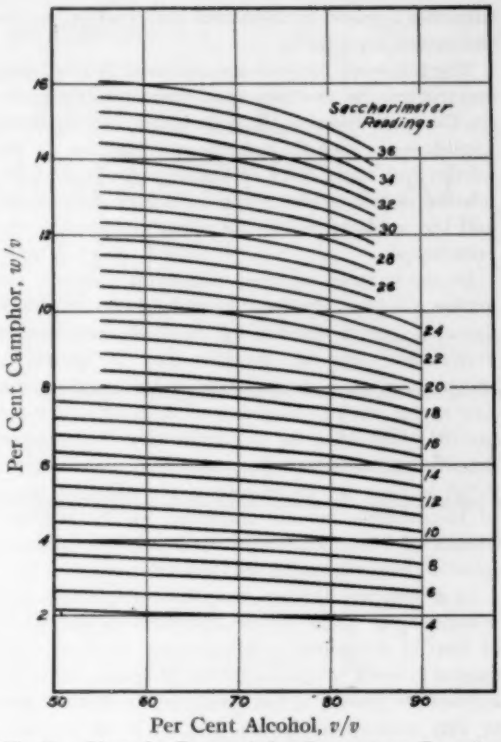

Fig. 7.—Chart for Determining Percentage Camphor from Saccharimeter Reading and Percentage Alcohol

looks upward on the graph until the reading, which, for example, was 21.5, is reached. Then, by referring to the ordinates, it is found that these values correspond to 9.74% camphor. An enlarged chart similar to Chart 7 should be prepared by each analyst showing points for a larger number of different percentages of camphor and alcohol.

By means of this modified method, several samples of spirit of camphor were assayed, and the results obtained are shown in Table VII. The data in this table show that the results are much more accurate than those obtained by the U. S. P. X method. In no instance is the result of an assay more than 0.20% off when compared with the theoretical value. The average error in the percentage of camphor obtained was −0.02%. The errors varied from −0.15% to +0.08%.

CONCLUSIONS

1. The method for the determination of camphor in spirit of camphor as given in the U. S. P. X has been found to give misleading results.

2. A method has been proposed making use of the percentage of alcohol as determined and of the saccharimeter readings. The percentages of camphor are read from a chart.

3. The proposed method has been found to give satisfactory results.

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(1) Poe, C. F., and Plein, E. M., *J. Phys. Chem.*, 37 (1934), 883.

Book Reviews

Optical Methods of Chemical Analysis, by THOMAS R. P. GIBB, JR., Ph.D. McGraw-Hill Book Company, New York, 1942. xiv + 391 pp., 299 illus., 15 x 23 cm. Price, $5.00.

This book, another member of the *International Chemical Series*, was designed by the author to give an abridged survey of the basic principles of optical methods of analysis. The author has deliberately emphasized the design and manipulation of optical instruments at the expense of an extensive discussion of theory. This does not mean that basic theory was neglected, for always may be found the mathematical and physical concepts upon which optical analysis is based. The extensive bibliography

furnishes a guide to additional information on the theoretical aspects.

The following subjects are reviewed in this book: spectrochemical analysis, spectrophotometric analysis, the applications of the colorimeter, turbidimeter, nephelometer and fluorphotometer, the use of the microscope, elementary crystallography and identification of crystals with the polarizing microscope, and the applications of the refractometer and the polariscope.

In the chapter on spectrochemical analysis, the author discusses the basic considerations of atomic emission spectra followed by the methods of sample excitations. Careful consideration is given to the comparison between spark and arc excitations, and useful wiring diagrams of apparatus are appended. The basis for the application of prism and grating spectroscopes is considered in sufficient detail so that the limitations of the different types of instruments become apparent to the student. Means of identification of elements by measurement of the wave length are then discussed.

In a separate section, quantitative spectroscopy is considered. This section embraces the principles of relative intensities and log-sector methods. Of special interest is the discussion of densitometry as applied to photographic emulsions. At the close of this section, as is the case with all sections, practical laboratory directions are given for the student and this is followed by a series of problems and discussion questions.

The polariscope, refractometer, microscope and others of these optical instruments are used frequently in pharmaceutical analysis and at times given recognition in official compendia. No doubt other optical equipment described in this text will become important in pharmaceutical research.

The chapter on "Identifying Crystals with the Polarizing Microscope" is very comprehensive for a necessarily limited discussion. It begins with a discussion of means of determining the refractive index of solids and liquids under the microscope by the Becke half-shadow method and other methods. This is followed by the application of the Nicol prism to the determination of extinction of anisotropic crystals. Approximately 20 pages are given over to a discussion of the more complex biaxial interference figures. The supplementary tables of refractive indices at the end of this chapter, while not complete, will prove useful.

Although this book was written primarily as a text for a course in optical analysis, it will prove to be a valuable auxiliary text in courses in pharmaceutical analysis and a reference book in this expanding field. The book is clearly written and the workmanship is commendatory. To be noted especially are the many clear diagrams of laboratory instruments.—M. W. GREEN.

The Interaction of Drugs and Cell Catalysts, by FREDERICK BERNHEIM. Burgess Publishing Company, Minneapolis, Minn., 1942. 85 pp., 9 figs., 21 x 28 cm. Price, $2.25.

It has been said that there is no end to the making of books, but it is rarely that a book is written in any field which presents a new viewpoint. In the field of pharmacology, such a book was A. J. Clark's "Cellular Pharmacology" and this book by Bernheim is worthy of consideration as a companion volume. While it was the purpose of Clark to use quantitative relationships between certain drugs and enzymes as a criterion for determining whether the drug is acting on cell surfaces or on cell catalysts, Dr. Bernheim's book is functioning to compile data to answer two questions: (*a*) if a drug alters the activity of a specific enzyme *in vitro*, how far can pharmacological effects be explained on this basis, and (*b*) if an enzyme inactivates a drug *in vitro*, can this explain its fate in the body?

The quest for the answer to these questions is an arduous one, but there are many data in the literature which can answer these questions in part. In fact the Bernheims and their collaborators have given much of this information to pharmacology. But the findings are still incomplete and at times inconsistent. The author has done a splendid job of evaluating and coördinating these data.

Part I of Dr. Bernheim's book discusses "Drugs That Affect the Activity of Certain Enzymes." The bulk of this section is given over to a discussion of drugs affecting cholinesterase. Eserine and eserine-like compounds, morphine, strychnine, curare and methylene blue are described in their relation to cholinergic and anticholinesterase activity. Cyanide, selenide, arsenic, copper, sulfanilamide and salicylate are considered in relation to other enzyme systems in this section.

Part II is given over to the discussion of "Drugs That Are Acted Upon by Certain Enzymes." In this section acetanilid and related compounds, histamine, atropine, acetyl derivatives of morphine, adrenaline and alcohol are discussed in relation to their metabolism in specific enzyme systems.

In this section the reviewer was particularly interested in the chapter on the metabolism of epinephrine. The author points out thatt here are three views concerning the possible mode of destruction of epinephrine: oxidation of the hydroxyl groups on the ring probably by means of the cytochrome system, oxidative deamination, and coupling of the phenolic groups with sulfate. The evidence for the three methods is very logically presented and the conclusion that "it may be said that there are a number of catalysts present in the body that can change adrenaline into a pharmacologically inactive compound. It is possible that all of them are involved, one being more active in one tissue, one in another. It is impossible from the present evidence to exclude any or to state that one mechanism predominates" seems to the reviewer to be a wise summary of the situation.

This excellent review will prove to be very stimulating to pharmacologists with imagination and foresight.—M. W. GREEN.